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# A BACTERIAL ROOT AND STEM DISEASE OF GUAYULE

W. A. CAMPBELL

(Accepted for publication December 22, 1946)

## INTRODUCTION

In May, 1944, a root disease of guayule (*Parthenium argentatum* A. Gray), with symptoms differing from those caused by *Phytophthora drechsleri* Tucker,[1] was first observed in an irrigated planting near Bakersfield, California. This disease, then termed resinous stem and root rot[2] on the basis of the characteristic black resinous exudate present on diseased roots and stems, has since been demonstrated to be caused by a bacterium and has been briefly described by Campbell and Presley[3] as a bacterial rot of guayule.

During the summer and fall of 1944 the disease was observed in several irrigated plantings in the San Joaquin Valley, California, but was an important cause of loss only in the field where it was first discovered. Diseased plants with similar symptoms were also observed in 1944 on several irrigated indicator plots in Texas. An outbreak of the disease occurred near Patterson, California, in August, 1945. In the same area severe losses from Phytophthora root rot also developed following midsummer irrigations. In some plantations on heavy soils, the combined loss from bacterial root and stem disease and Phytophthora root rot was 20 per cent of the existing stand. The bacterial disease also caused severe losses in several plantings near Bakersfield following heavy irrigations in July and August, 1945.

Losses from bacterial root and stem disease in several irrigated fields occurred mainly in localized areas, which because of topography or location received the most water. In these fields moisture conditions were favorable for the development and dissemination of the pathogen only in low places or at the beginning or end of the irrigation runs where water accumulated. In other fields on soils of good water-holding capacity, the loss was more uniformly distributed and followed heavy irrigations during hot summer weather.

## SYMPTOMS

In common with other root diseases of guayule, wilting is usually the first readily recognized symptom of bacterial root and stem disease. The degree and completeness of wilting are determined by the vegetative condition of the plants when attacked and by weather conditions. The bacterium in-

[1] Braun, A. J. Phytophthora root rot of guayule. (Abstr.) Phytopath. 34: 933. 1944.

[2] Guayule plantation diseases and their control. Mimeograph release. Special Guayule Research Project. 19pp. 1944.

[3] Campbell, W. A., and J. T. Presley. Diseases of cultivated guayule and their control. U. S. Dept. Agr. Circ. 749. 1946.

vades the root tissue of succulent plants and, consequently, affected plants wilt suddenly, without recovery, during hot weather. Nonsucculent plants frequently exhibit progressive wilting of the branches when attacked. Root lesions develop more slowly than in succulent plants and cause progressive interference with water uptake, and wilting may take place branch by branch. The plants may recover during the night and retain their normal appearance for several days before they permanently wilt. In cool weather diseased nonsucculent plants often do not wilt. Instead the leaves gradually yellow and dry on the plant.

The bark on the lower part of the main branches of recently wilted plants, or those that have been dead for a short time, usually has a brownish or blackish resinous exudate. The amount and distribution of this vary considerably from one diseased plant to another but its presence is a characteristic symptom of bacterial disease. The resinous exudate follows the extension of the lesions into the branches and is at first yellowish or light brown but quickly turns dark brown and eventually black. In the early stage of stem lesions the resin exudate is present as small droplets but these gradually increase in size and amount until the bark is often covered with an extensive resinous coating (Fig. 1, A).

When a diseased plant is pulled from the ground, the root crown and upper tap root usually are covered with an adhering layer of resin and earth (Fig. 1, B). The root lesions differ considerably from those caused by *Phytophthora drechsleri* inasmuch as the diseased tissue beneath the lesion does not become blackened or sunken. Except in the advanced stages following invasion by secondary organisms, the diseased root cortex underneath the resin-covered bark is usually but slightly discolored. Diseased cortex is, however, softer than healthy root tissue and somewhat cheesy in texture. After a few seconds' exposure to the air, the cut surface turns a dirty red or pink before finally becoming brown, in sharp contrast to the olive-green oxidation color imparted to healthy root tissue under the same conditions. Older diseased tissue usually is a dull red-brown but may be almost black in the final stages of disintegration. The pink pigment of the diseased root tissue in its early stages is water soluble and quickly diffuses out when such tissue is placed in water. A pronounced pink or red discoloration is usually present in the cambium and newly formed wood for some distance in advance of the lesions.

On two- and three-year-old plants that are not abundantly supplied with water throughout the summer but are irrigated heavily but once or twice during the season, root lesions may develop to a limited extent. These may be checked by drying but again become active on subsequent irrigations. Therefore losses developing in an area are usually only a fraction of the eventual loss since further mortality generally takes place following irrigations of moderate amounts. In all observed instances of severe loss from bacterial disease the original outbreak followed heavy irrigations.

ORGANISMS ASSOCIATED WITH BACTERIAL DISEASE

During the two growing seasons that the disease has been under observation numerous isolations have been made from root and stem lesions.[4] A

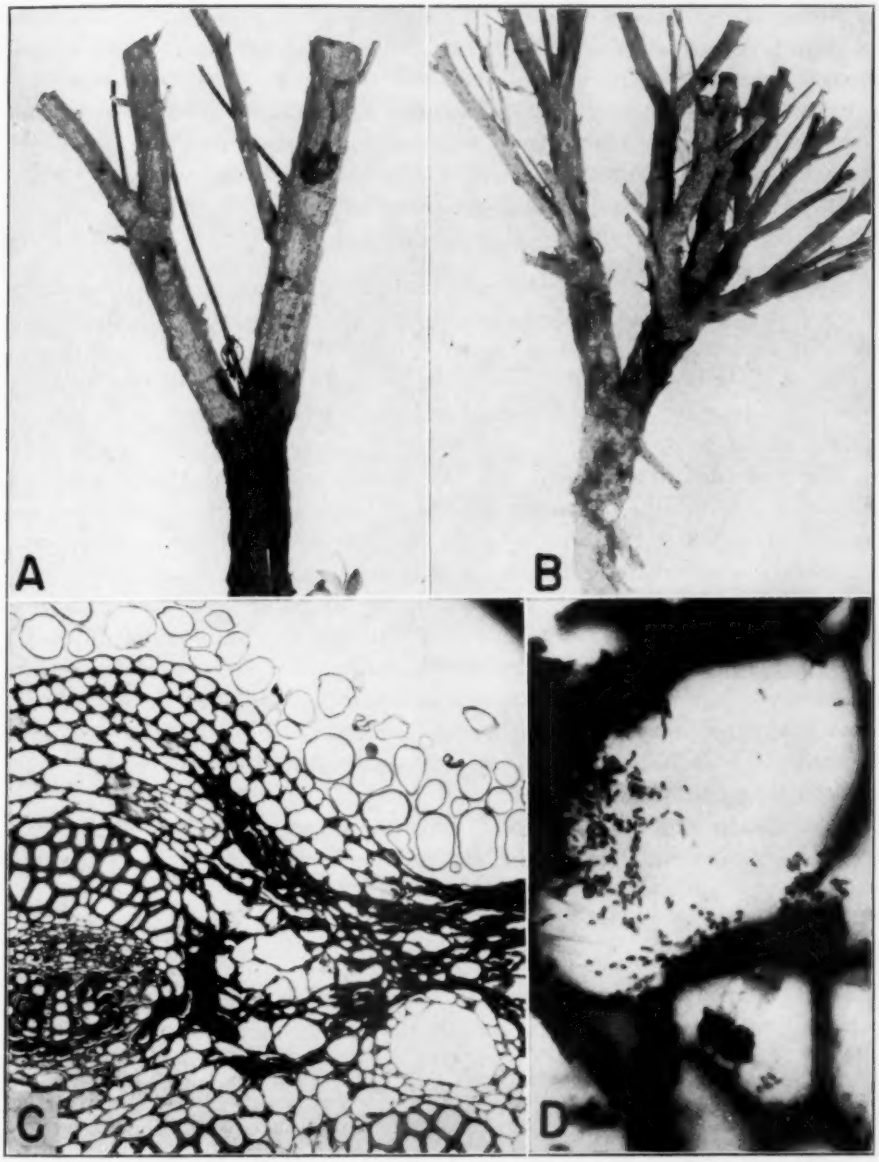


FIG. 1. Bacterial root and stem disease of guayule caused by *Erwinia sp.* A. Resinous exudate on stem. Approximately natural size. B. Resinous exudate on root crown, tap root, and stems of 3-year-old diseased plant. Approximately $\frac{1}{3}$ natural size. C. Cross-section of diseased stem showing breakdown of cortical cells. D. Bacteria in diseased cells.

[4] Many of the isolations were made by Lois Weston Weeth and A. J. Braun.

variety of fungi were commonly obtained from lesions at the root crown or below. These include two species of *Fusarium*, *Alternaria sp.*, and several representatives of the *Mucorales*. None of these fungi were pathogenic to guayule in inoculation experiments in the greenhouse.

Bacteria were also present in the root lesions but were usually obscured by rapidly growing fungi. Isolations from the uppermost lesions in the stems invariably gave pure cultures of the bacterium. The presence of this organism in diseased cortical tissues could also be demonstrated by microscopic examination. The guayule bacterium has been identified by Starr[5] as an *Erwinia sp.*

## INOCULATION EXPERIMENTS

### *Proof of Pathogenicity*

Repeated inoculation experiments have demonstrated the pathogenicity of the *Erwinia sp.*, isolated from guayule, to guayule seedlings in the greenhouse at temperatures ranging from 70° to 90° F. In a representative experiment eight 6-month-old transplants, 4 plants to a can, in two 5-gallon cans of pasteurized soil were inoculated on October 26, 1945, with a pure culture of the bacterium grown on potato-dextrose agar. Inoculation was by means of a longitudinal cut, one-half inch long, through the bark at and just below ground line. A small amount of bacteria and agar was placed in the cut, after which cotton was bound over the area and the soil replaced. The checks were treated similarly with sterile potato-dextrose agar. Following inoculation the plants were saturated for 24 hr. and watered 2 or 3 times a day thereafter. Five out of 8 inoculated plants were killed by November 16, and the remaining three were pulled on that date for examination. Two of these had large lesions on the roots but the leaves had not wilted; the third had a small root lesion that had not progressed into the stem. The bacterium was recovered in pure culture from diseased tissue in the stems. No other organism was present. The four check plants remained healthy and when the roots were examined approximately 3 weeks after inoculation with plain agar, the wounds were found to be completely healed.

### *Root Injuries and Infection*

In the foregoing experiment the bacterium was introduced into the root tissue through a cut in the bark and in massive amounts. In another experiment to determine whether or not the bacterium could penetrate uninjured bark, 8 plants were inoculated by applying bacteria on potato-dextrose agar directly to uninjured bark just below the root crown. Four check plants were similarly treated with plain agar. Cotton was placed over the inoculations and the soil was pressed over the cotton. The soil in the cans was kept continuously soaked for 24 hr. following inoculation and watered twice a day thereafter.

After two months 3 of the inoculated plants had definite root lesions,

[5] Starr, M. P. The casual agent of bacterial root and stem disease of guayule. Phytopath. **37**: 291–300. 1947.

which extended into the stems. Five plants remained healthy as did the 4 check plants inoculated with plain agar.

In further experiments on the relation of wounds to infection, 6-month-old plants growing 4 to a 5-gallon can were used. In each can 2 of the 4 plants were injured just below ground line by several small cuts made in the bark by a sharp scalpel and 2 were left uninjured. Inoculation was by means of a suspension of bacteria in distilled water, followed by heavy watering for 24 hr. and twice a day thereafter. The check plants were flooded with distilled water immediately after wounding.

The four plants injured at the ground line and flooded with a suspension of bacteria in water wilted in from 1 to 2 weeks after inoculation. The four noninjured plants remained healthy as did the injured and noninjured check plants. The wounds on the roots of the injured checks had healed in 3 weeks at which time all of the plants were pulled for examination.

In a repetition of the same experiment with 8 succulent and 8 hardened plants, 15 out of 16 plants in both categories with injuries at the crown and flooded with bacteria succumbed to the disease and 1 remained uninfected at the end of 2 weeks. Two noninjured hardened plants out of 8 inoculated became infected, and all 8 inoculated noninjured succulent plants remained healthy. There appeared to be little difference in the susceptibility of injured succulent and hardened plants under the conditions of the experiment. However, the succulent plants wilted more quickly than the hardened plants when attacked.

### *Temperature and Infection*

In order to determine the effect of temperature upon the pathogenicity of *Erwinia sp.* from guayule, inoculations were made on 7-month-old guayule seedlings at 9 constant temperatures ranging from 55° to 98° F. All of the plants used were of variety 593 and were in approximately the same degree of succulence when placed in the temperature tanks. Six plants were maintained at each temperature. These were inoculated on January 11 by applying a half-inch square portion of isolate 263C on nutrient-dextrose agar directly to small cuts through the bark 1 inch below the soil surface. After inoculation the plants were watered at frequent intervals in order to keep the soil continuously moist. The water was heated or cooled to the proper temperature before applying. The results of the experiment are summarized in table 1.

The bacterium from guayule readily caused infection of guayule at temperatures from 70° to 98° F. Two plants out of 6 were infected at 64°, indicating limited ability to cause infection at this temperature during a period of 14 days. At both 55° and 60° the inoculation wounds were either healed or were healing. Judging from the rapidity with which external disease expression developed as exemplified by wilting, the guayule bacterium was most virulent from 75° to 85° inclusive. The slowness of disease expression at 70° was due to the lower transpiration rate at that

temperature and consequently the leaves did not wilt until the cortex was badly decayed. At 89° and 98° the plants had become somewhat hardened and consequently wilting was slower and less evident than at the temperatures at which the plants were growing more vigorously. In addition, at these high temperatures the disease was confined to the root and the bacteria did not invade the stem except for a short distance above the ground. At lower temperatures the cortex of the branches was softened even to the ends of the branches before permanent wilting occurred.

Check plants grown at the same temperatures and inoculated with nutrient-dextrose agar remained healthy during the 14 days. In most plants the inoculation wounds had completely healed.

### *Tissue Affected*

In order to determine the stem tissues invaded by the bacterium sections for microscopic examination were taken at various heights on the stems of diseased plants. These were fixed in formalin–propionic acid–ethanol solu-

TABLE 1.—*Effect of constant temperatures upon the time required for infection of guayule by Erwinia sp. isolated from guayule*

| Soil temperature[a] | Total plants inoculated | Plants diseased in 14 days | Time required for first disease expression[b] | Average time for complete wilting per plant |
|---|---|---|---|---|
| ° *F.* | *Number* | *Number* | *Days* | *Days* |
| 55 | 6 | 0 | 0 | ..... |
| 60 | 6 | 0 | 0 | ..... |
| 64 | 6 | 2 | 7 | ..... |
| 70 | 6 | 6 | 8 | 13 |
| 75 | 6 | 6 | 5 | 7 |
| 80 | 6 | 6 | 4 | 7 |
| 85 | 6 | 6 | 6 | 8 |
| 89 | 6 | 6 | 9 | 12 |
| 98 | 6 | 6 | 11 | ..... |

[a] At one inch depth, range plus or minus 1° F.
[b] As indicated by slight wilting of leaves.

tion, embedded in paraffin, sectioned, and stained by the usual histological methods.[6] Because root tissues quickly became badly disorganized following infection, the histological studies were confined to the stems.

The bacterium invades primarily the cortex, causing a breakdown of the cortical parenchyma (Fig. 1, C). The resin flow commonly observed on diseased stems resulted from the breakdown of the cells around the resin canals and consequent release of their contents, which escaped to the outside through the disintegrated cortical tissue. The bacterium could be readily demonstrated in large numbers in the diseased cortex (Fig. 1, D). The upward progress of the bacteria through the cortex took place through intercellular spaces and by passage from one diseased cell to another.

[6] The author is greatly indebted to L. C. Erickson for the histological sections and photomicrographs.

SUMMARY

A bacterial root and stem disease of guayule caused by an *Erwinia sp.* caused considerable loss in irrigated plantations in the San Joaquin Valley of California in 1944 and 1945. Losses from bacterial disease were generally associated with heavy summer irrigations. In several fields losses were restricted to low places where water accumulated but in others the losses were more uniformly distributed over the area.

In the field, infections developed at or below the root crown, presumably through injuries of various kinds. Root and stem lesions were frequently but not always accompanied by copious resinous exudation. Symptom expression was influenced by temperature and type of growth. Succulent plants wilted suddenly but hardened plants frequently wilted branch by branch or the leaves dried on the plant without pronounced wilting.

The pathogenicity of the organism was demonstrated by inoculation experiments in the greenhouse. The average time required for complete wilting of inoculated plants at constant temperatures of 75°, 80°, and 85° F. was 7, 7, and 8 days, respectively. Disease expression was slower at 70°, 89°, and 98° F. Below 60° F. the inoculation wounds healed without infection.

Histological studies of diseased stems demonstrated that the bacteria were confined to the cortical cells. These quickly disintegrated in the presence of the bacteria and permitted the bark to separate readily from the stems.

SPECIAL GUAYULE RESEARCH PROJECT,
SALINAS, CALIFORNIA.

# DODDER AS AN AID IN TESTING SOME PLANT SPECIES FOR CURLY-TOP VIRUS

N. J. GIDDINGS[1]

(Accepted for publication January 4, 1947)

Some plant species highly susceptible to curly top are very unsatisfactory hosts for the beet leafhopper (*Eutettix tenellus* Baker). Several species of *Nicotiana* and the commercial varieties of tomato are in such a grouping. The leafhoppers get their legs and wings entangled with the hairs or by the sticky exudate and may live only two or three days under warm, favorable, feeding conditions. During the time that they are caged on such plants they are not likely to feed well.

It has been found that one or more species of dodder thrive well upon such plants and that the leafhoppers feed well upon dodder. The leafhoppers also survive very well in cages which include a reasonably strong growth of dodder and not many leaves of the plant that is an undesirable host for them. The dodder takes up curly-top virus along with nutrients from its host, as reported by Bennett,[2] and the leafhoppers readily pick up the virus from the dodder. The method is illustrated by the pictures in figure 1.

During 1938–39 several groups of previously inoculated Turkish tobacco plants were tested for curly-top virus using young sugar beets as test plants and one leafhopper per plant for inoculation. Five groups were tested without the aid of dodder and three groups with *Cuscuta subinclusa* Dur. and Hilg. The results are given in table 1.

TABLE 1.—*Results of tests for curly-top virus in inoculated plants of Nicotiana tabacum L.*

| Without dodder | | | | With dodder | | | |
|---|---|---|---|---|---|---|---|
| Source plants | Test plants | | | Source plants | Test plants | | |
| | Inoculated | Diseased | | | Inoculated | Diseased | |
| *No.* | *No.* | *No.* | *Pct.* | *No.* | *No.* | *No.* | *Pct.* |
| 8 | 59 | 5 | 8 | 4 | 60 | 30 | 50 |
| 7 | 44 | 4 | 9 | | | | |
| 16 | 189 | 35 | 19 | 11 | 126 | 65 | 52 |
| 9 | 85 | 25 | 29 | | | | |
| 9 | 108 | 29 | 27 | 9 | 108 | 59 | 55 |
| Tot. 49 | 485 | 98 | ..... | 24 | 294 | 154 | ..... |
| Av. Pct. | ..... | ..... | 20 | ..... | ..... | ..... | 52 |

[1] Senior Pathologist, Division of Sugar Plant Investigations, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, United States Department of Agriculture.

[2] Bennett, C. W. Dodder transmission of plant viruses. Phytopath. **34**: 905–932. 1944.

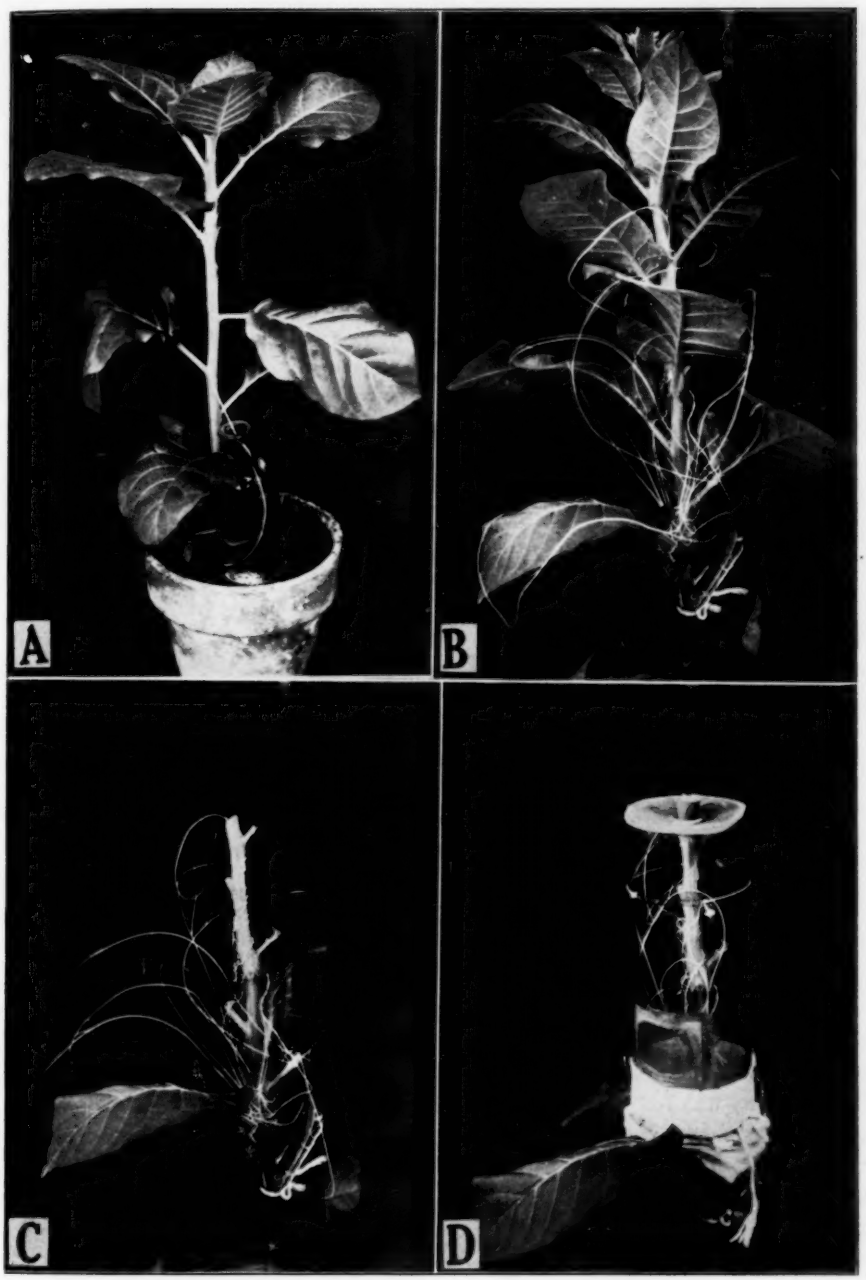


FIG. 1. The use of dodder on *Nicotiana tabacum* L. A. Strand of dodder in bottle of water just getting established upon tobacco. B. Dodder growing vigorously on tobacco. C. Tip and most of adjacent leaves cut off so that leafhoppers will have less interference from them while feeding on dodder. Cotton will be wrapped around tobacco stem just above string. D. Celluloid cage tied on over dodder.

Of the 49 plants tested without dodder, 45 per cent failed to give infection in any of the test plants, while among the 24 plants tested with dodder, 25 per cent failed to give infection in any of the test plants. There was more than two and one-half times as much infection among the test plants when dodder was used on the tobacco than when the test was made using leafhoppers which had to feed directly on the tobacco.

During 1939 a comparative test was made using inoculated plants of *Nicotiana glutinosa* L. In this experiment, the same plants were first tested without the dodder and then with *Cuscuta subinclusa*. The results are given in table 2.

TABLE 2.—*Results of tests for curly-top virus in inoculated plants of Nicotiana glutinosa L., 1939*

| Source plant number | Without dodder | | | With dodder | | |
|---|---|---|---|---|---|---|
| | Test plants | | | Test plants | | |
| | Inoculated[a] | Diseased | | Inoculated[b] | Diseased | |
| | *No.* | *No.* | *Pct.* | *No.* | *No.* | *Pct.* |
| 82 | 15 | 1 | 7 | 12 | 5 | 42 |
| 83 | 21 | 1 | 5 | 12 | 10 | 83 |
| 84 | 15 | 0 | 0 | 12 | 0 | 0 |
| 85 | 25 | 2 | 8 | 12 | 5 | 42 |
| 86 | 23 | 1 | 4 | 12 | 8 | 67 |
| 87 | 22 | 0 | 0 | 4 | 1 | 25 |
| 88 | 20 | 10 | 50 | 11 | 11 | 100 |
| 89 | 32 | 0 | 0 | 12 | 10 | 83 |
| 90 | 22 | 2 | 9 | 12 | 10 | 83 |
| 91 | 20 | 2 | 50 | 12 | 11 | 92 |
| 92 | 24 | 5 | 21 | 12 | 11 | 92 |
| 93 | 19 | 6 | 32 | 12 | 12 | 100 |
| 95 | 12 | 0 | 0 | 10 | 4 | 40 |
| 113 | 12 | 8 | 67 | 11 | 9 | 82 |
| Totals | 282 | 38 | ..... | 156 | 108 | ..... |
| Av. Pct. | ..... | ..... | 13 | ..... | ..... | 69 |

[a] Test plants were inoculated July 10, 1939.
[b] Test plants were inoculated August 11, 1939.

Three of the *Nicotiana glutinosa* plants gave negative tests without the dodder, but positive tests when dodder was used. There was more than five times as much infection among sugar beet test plants when dodder was used than when it was not used.

The method has been used many times since the above comparisons were made, and it has been very satisfactory if care was exercised to see that the dodder was well established and growing vigorously upon the host plant to be tested.

U. S. DEPARTMENT OF AGRICULTURE,
RIVERSIDE, CALIFORNIA.

# STUDIES ON FACTORS AFFECTING THE INFECTIVITY OF HELMINTHOSPORIUM ORYZAE[1]

ARDEN F. SHERF, ROBERT M. PAGE, E. C. TULLIS, AND THOMAS L. MORGAN[2]

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*Helminthosporium oryzae* van Breda de Haan, the cause of the brown-spot disease of rice, is a fungus of widespread distribution and occurs in every rice-growing country. The disease may cause extensive damage to seed-bed rice by damping-off the seedlings. On older plants, the leaf lesions produced by *H. oryzae* may impair the yield by reducing the effective photosynthetic areas of the leaves. On mature plants, damage to rice kernels reduces quality as well as yield of rice.

The macroscopic symptoms of the brown-spot disease have been described in detail by Nisikado, Nogaka-Tokugyoshi, and Miyake (6), and Tullis (8) reported the histological features of the disease. Hemmi and his coworkers (1, 2) studied the influence of temperature and moisture on the initiation and severity of the disease. The effects of other environmental factors on the host-parasite relationship were investigated by Suzuki (7).

All of these studies on the influence of environmental factors, however, have been with oriental collections of *Helminthosporium oryzae,* and there is some possibility that there is a strain difference between collections made in the Orient and in the United States (4). The widespread distribution and damage caused by the disease justify a further investigation of the factors affecting the disease syndrome.

## MATERIALS AND METHODS

The susceptible rice varieties Onsen and Butte were selected for experimental use because it was possible to obtain seed free of the disease. When other varieties, the seeds of which were diseased, were used, they were subjected to the hot-water treatment as recommended by Nisikado and Miyake (5). For most work in the greenhouse the rice was grown in one-half-gallon glazed pots. The seedlings were routinely thinned to 15 per pot. For experiments in which the rice was grown to maturity, one-gallon pots with five plants per pot were employed.

The inoculum was of two types: a dust of conidia and a dust of ground vegetative mycelium. The conidia were produced on moist sterilized oats or rye. Three hundred grams of seeds, placed in a Fernbach flask with 345 ml. of water, were steamed for one-half hour, autoclaved for 20 min. at

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15 lb. pressure, allowed to stand over night, and re-autoclaved. When the flasks had cooled, they were inoculated with an aqueous suspension of conidia and incubated at 22–24° C. in the dark for 14 days. Three to five 200-ml. portions of water were added singly to the flasks, vigorously shaken, and the resulting suspension of conidia removed from the substratum by straining through wire gauze into cylinders after each such addition. The cylinders were allowed to stand overnight in a room at 8° C. to allow the conidia to settle. The supernatant liquid was decanted, and the concentrated conidia were collected in a Buchner funnel on coarse filter paper. The filter cake was broken up and dried in a steam oven for 24 hr. at 40° C. Approximately 3–4 g. of conidia, 96–97 per cent of which were capable of germinating, were obtained in this way from each 300-g. sample of dry substrate.

The mycelial dust was obtained by growing *Helminthosporium oryzae* in aerated liquid culture. Four-liter Pyrex serum storage bottles were used as culture vessels, with three liters of a medium composed of a solution of 10 per cent (by volume) black-strap molasses and 0.5 per cent peptone in each bottle. After sterilization at 15 lb. pressure for 20 min. and cooling, the bottles were inoculated with approximately two ml. of an aqueous suspension of conidia. During the incubation period of six days at 27–31° C. the cultures were aerated with sterile air introduced at a rate of 1500 ml./min. The mycelium was collected in a Buchner funnel, the filter cake broken up and dried at 40° C. for 24 hr. The resulting product was ground in a Wiley mill to produce a dust. Both the conidia and the ground mycelium were mixed with a filler of limestone (500 mesh) immediately before use.

Rice plants to be inoculated in the greenhouse were first moistened with water from an atomizer. The pots were then placed in a wooden cabinet and the inoculum applied with a small duster improvised for the purpose. In the field the dust was applied in the same way, except that a portable cloth screen was substituted for the cabinet to minimize drifting of the dust. After the plants were dusted in the greenhouse they were placed in a humidity tent which consisted of a muslin-covered pipe framework erected over a greenhouse bench. The humidity was maintained at a high level by a humidifier that was provided with a time switch, so that the intervals during which the humidifier was in operation could be adjusted. A spray of water on the top of the tent aided in the maintenance of high humidity.

The evaluation of results in terms of degree of infection presented a problem somewhat difficult of solution. In some cases the number of lesions per leaf produced by a given weight of inoculum applied to a known area was used as a criterion. In other cases, leaves were examined under the microscope after infection had developed, and the number of conidia adhering to the leaves was compared with the number of lesions. A third method was to estimate the degree of infection and express it in terms of heavy, moderate, or light infection. A refinement of the latter procedure was to compare the infected leaves with a photographic standard showing leaves with various degrees of infection expressed as the area of the leaf which was

covered with lesions. None of these methods was entirely satisfactory, but together they gave a practical index whereby the results of individual experiments could be evaluated and interpreted.

### MOISTURE RELATIONS

Experiments on the moisture relations of the host and parasite fell into two divisions: (1) the effect of moisture prior to inoculation and (2) the effect of moisture after inoculation. Two experiments were conducted to ascertain the effect of water prior to inoculation.

In order to determine whether rice plants could be predisposed to infection by *Helminthosporium oryzae*, by exposing them to conditions of high relative humidity before inoculation with the pathogen, three pots of rice (var. Butte) were placed in the humidity tent for 24 hr. Three controls were kept on the greenhouse bench. Both the controls and the plants that had been kept at a high humidity were inoculated with a dust of conidia and placed in the humidity tent for 24 hr. They were then removed and allowed to stand on the greenhouse bench for three days. There were no detectable differences in the degree of infection between the plants kept at high humidity before inoculation and the control plants.

The presence of free water on the leaves of the host at the time of inoculation appeared to favor infection. Duplicate pots of rice (var. Butte) were treated as follows: two sets of pots were placed in the humidity tent for 1 hr., after which they were removed and sprayed with water from an atomizer until the leaves of the plants were thoroughly wet. They were then inoculated with a dust of conidia. One set was placed on the greenhouse bench, while the other was placed in the humidity tent together with a third set that had been inoculated while the leaves were dry. After three days the plants placed on the greenhouse bench after inoculation had no infection, while all of the plants that had been in the humidity tent for 24 hr. developed leaf lesions. The plants the leaves of which had been moistened before inoculation had more lesions, and the infection was considerably more severe than on the plants that had been dusted with conidia while the leaves were dry. An experiment in which duplicate pots of rice (var. Early Wataribune) were dusted with ground mycelium gave similar results. Free moisture on the leaves obviously had favored infection. The results of a field experiment to be described in a later section supported this conclusion.

Whether free moisture on the leaves subsequent to inoculation was necessary for infection was not determined. It has been reported (3) that infection would take place if the plants were exposed for 18 hr. to 92 per cent relative humidity at 25° C. Hemmi and Nojima (1) found the period of wetness necessary for infection to be 8 hr. at 20° C., 4 hr. at 25° C. and 30° C., and 6 hr. at 35° C. In an experiment in which 15-day-old rice plants were inoculated with a conidial dust and placed in the humidity tent, it was found by removing duplicate pots at 2-hr. intervals that a period of 10 hr. of high humidity was necessary for the establishment of optimum infection at 22° C.

In order to determine how long conidia would remain viable on the leaves of rice plants until conditions were favorable for the initiation of disease, 22 pots of rice (var. Caloro) were inoculated with conidia and placed on the greenhouse bench. At 2-day intervals up to 18 days and on the thirtieth day duplicate pots were placed in the humidity tent for a 24-hr. period. Infection developed on all the plants except those which were placed in the humidity tent 30 days after inoculation. These results indicated that the conidia remained viable at least 18 days. The 30-day plants were not infected because all leaves present at the time of inoculation had shrivelled

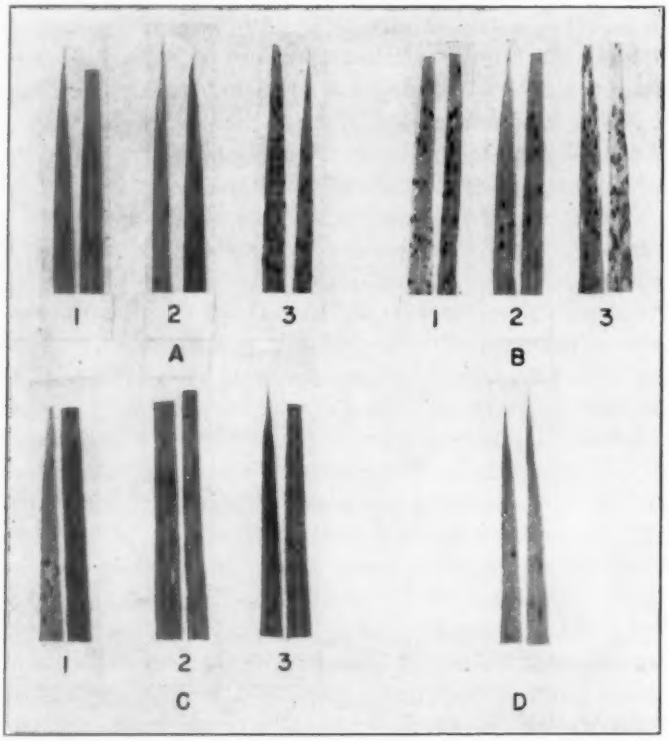


FIG. 1. Effect of time of inoculation on severity of infection of rice by *Helminthosporium oryzae*. Leaves from plants dusted with inoculum on first day (A), on second day (B), and on third day (C). Leaves from control plots (D). Morning inoculations (1), noon inoculations (2), and evening inoculations (3). Leaves were collected at random from separate plots.

naturally. A second experiment extended the time to 28 days, at which time lesions could be distinguished at the tips of the oldest leaves. From these results it would appear that in the greenhouse the conidia remain in a viable state on the leaves for the life of the leaf. In the field this probably would not hold true, because wind and rain would tend to dislodge the conidia from the leaves, and in many cases environmental conditions would be unfavorable for the retention of viability.

Although the presence of water is a requisite to the initiation of infection, large amounts of water in the form of rain tend to reduce the amount of infection. The effect of heavy rainfall upon freshly inoculated plants was tested by exposing them to a simulated rain in which $\frac{3}{16}$ of an inch fell in a 5-min. period. These plants together with unwashed, inoculated controls were kept in the humidity tent 24 hr. No infection developed on the washed plants, but abundant lesions appeared on the leaves of the inoculated controls. It would appear, therefore, that a hard rain immediately following inoculation will effectively wash the conidia from the leaves before penetration can be effected. A subsequent field test indicated that even a light rain washes many of the conidia from the plants and results in a reduced amount of infection.

### AIR TEMPERATURE

The influence of air temperature during the infection period upon the severity of infection was investigated. Triplicate pots of rice (var. Onsen) were inoculated with a dust of conidia and placed in large sheet-metal baskets immersed in water baths adjusted to 15°, 20°, 25°, 30°, and 35° C. The baskets were covered, and a thermometer inserted through the lid of each. The air temperatures within the baskets agreed closely with the temperatures of the water baths. A high level of moisture was obtained by keeping a layer of water in the baskets and by frequent atomization with water. After 24 hr. in the baskets the plants were removed to the greenhouse bench. Representative leaves were removed and the number of conidia adhering to each leaf was compared with the number of lesions present on that leaf. The results (Table 1) indicated that the temperature range required for infection to become established was 20–30° C. No infection was observed at 15° C. and very little at 35° C. The lowest conidium/lesion ratios were found at 20° and 25° C., which indicated that the optimum range for infection was between 20° and 25° C. This test was repeated using ground mycelium as inoculum, and the results indicated that the over-all and optimum temperature ranges were the same as those required in the case of conidia.

TABLE 1.—*The effect of air temperature on the infectivity of the conidia of Helminthosporium oryzae*

| Temperature, ° C. | Conidia per leaf, number | Lesions per leaf, number | Ratio, conidia/lesions |
|---|---|---|---|
| 15 | 108 | 0 | ..... |
| 20 | 142 | 48 | 3 |
| 25 | 156 | 36 | 4.3 |
| 30 | 212 | 26 | 8 |
| 35 | 0 | 0 | ..... |

### TIME OF DAY

From the foregoing data it might be surmised that in the rice-growing areas of the southern United States the time of day for inoculation of rice

that would give the greatest amount of infection would be the evening. Moisture conditions then would be more favorable for penetration by germ tubes from conidia or mycelial fragments than at any other time of day. The temperature would ordinarily be within the optimum range. Conditions would be expected to remain favorable for a longer period of time.

To test this supposition a field experiment was conducted during the summer of 1945 at Beaumont, Texas. Test plots were four feet square and were selected from a field of 45-day-old rice (var. Onsen). Because the rice was planted late in the season (June 4), it was in the late tillering stage, although only approximately 18 inches high. At the time of inoculation the field was flooded with two to three inches of water. Each plot was dusted with 0.9 g. of mycelial dust (40 per cent viable) mixed with 1.6 g. of powdered limestone. Three applications, one in the morning, one near noon, and one in the evening were made on duplicate plots on three alternate days. These plots were observed one day and three days after the inoculations. The severity of infection was estimated by comparing the infected leaves with photographic standards previously prepared for reference and was expressed in terms of the area of the leaf surface that was covered with lesions (Table 2). In general, there was considerable variability between

TABLE 2.—*Effect of time of inoculation on the severity of infection of rice by Helminthosporium oryzae*

| Time of inoculation | | Condition of plants | Percentage of leaf covered with lesions | | Infection |
|---|---|---|---|---|---|
| Day | Hour | | Day 6 | Day 8 | |
| 1[a] | 6 : 30 a.m. | Dry | 0–1 | 0–1 | Light |
| | 2 : 30 p.m. | Dry | 0–1 | 0–1 | Light |
| | 8 : 30 p.m. | Wet | 10–15 | 10 | Moderate |
| 3[b] | 7 : 00 a.m. | Wet | 0–15 | 10 | Moderate |
| | 2 : 00 p.m. | Dry | 0–5 | 5 | Light |
| | 9 : 00 p.m. | Wet | 10–15 | 25 | Severe |
| 5 | 6 : 45 a.m. | Wet | ..... | 10 | Moderate |
| | 2 : 30 p.m. | Dry | ..... | 0–10 | Light |
| | 9 : 30 p.m. | Wet | ..... | 20–25 | Severe |

[a] Light showers at 3 : 30 p.m. and 7 : 30 p.m.
[b] Light shower at 7 : 30 p.m.

the plants within a plot, but when the plots were compared there was no difficulty in discerning differences between them. The leaf samples shown in figure 1 illustrate the difference between treatments.

It was concluded that evening inoculation gave consistently more severe infection than morning or noon application. Morning application produced more severe infection in those cases where the plants were wet at the time of inoculation (days three and five), and, in general, infection was more severe on the plants that were dusted while the leaves were wet. The difference between the amounts of infection resulting from the two o'clock inoculations on the first and third and on the fifth day may be accounted for by the fact

that the light rains on these two days washed much of the mycelial dust from the leaves. That less inoculum was washed from plants inoculated while wet is indicated by the fact that more severe infection resulted from the morning application on the third day than on the first.

### FORM OF THE PATHOGEN

Mycelial fragments were capable of initiating the brown-spot disease. Since the number of the mycelial fragments that resumed growth and served as inoculum was invariably lower than that of the viable conidia, it was necessary to apply per unit area a greater weight of the mycelium than of the conidia to produce a given amount of infection. In general, conidia were five to ten times more effective in producing infection than mycelial dust when the two were compared on a weight basis.

The infection produced by the mycelial fragments was similar to that produced by the conidia in every respect. The fragments resumed growth on the leaf surface within 24 hr., and the newly formed mycelial tube usually penetrated the leaf epidermis directly rather than through the stomata. Appressoria were often formed at the point of penetration.

### EFFECT OF VARIETY OF HOST

Unlike other leaf-spotting diseases brown spot appeared to meet no varietal barriers of resistance. Greenhouse tests with 20 rice varieties failed to demonstrate any measurable differences in resistance to this disease. This absence of varietal resistance is in contrast to reports of Japanese workers; however, it must be recalled their work was done with Oriental isolates of the pathogen as well as with Oriental varieties of rice. The reactions of Oriental isolates on American varieties or American isolates on Oriental varieties are not now known.

### EFFECT OF AGE OF HOST ON SUSCEPTIBILITY

In many plant diseases, the susceptibility of the host varies at different stages of growth. In order to test the susceptibility of rice of various ages to leaf infection by *Helminthosporium oryzae*, rice (var. Early Wataribune) was planted weekly for three weeks and fortnightly thereafter. When the oldest plants were 85 days old and had headed, the entire series of plants was inoculated with a dust of conidia. After 24 hr. in the humidity tent and 48 hr. on the greenhouse bench, the plants were examined. The youngest plants (15 days old) showed the least infection, but no differences in the severity of infection could be noted among the older plants. Leaf lesions and dead leaves were produced on plants of all ages. Perhaps the rigid, almost upright leaves of the youngest plants intercepted less of the inoculum than the more flexible leaves of the older plants. The fact that the infection of older plants was always most severe at the bend of a leaf where the surface was nearly horizontal would tend to support this interpretation. Heads

that were present at the time of inoculation were severely discolored and the spikelets were shrivelled.

A second test with Onsen rice substantiated the results of the first. Mycelial dust as well as conidia was used for inoculation, and a large number of lesions was produced on plants that were from two to four months old. There were no apparent differences in the relative infectivity of the conidia and mycelial fragments on rice at different stages of growth.

## DISCUSSION

The results of the present investigation indicate that the influence of environmental factors on the susceptibility of rice to brown-spot infection was an effect exerted on the pathogen rather than on the host, as is generally the case in leaf-spot host-parasite relationships. Rice plants could not be predisposed to infection by keeping them in a humid atmosphere prior to inoculation. This result would be expected, since microscopic examination of leaves indicated that direct penetration of the leaf epidermis occurred more frequently than entrance through the stomata. Factors such as humidity and light which affect stomatal opening would, therefore, not influence the susceptibility of the host to any marked degree. Conditions obtained in a rice field offer relatively uniform moisture and light effects on the host; therefore, the severity of brown-spot disease in any field is dependent upon the suitability of environmental factors to the dissemination of the pathogen and to its infection process. The organism is probably present in rice fields every year, but variations in weather, which have been demonstrated in this work to influence greatly conidial germination and infection, cause noticeable differences in severity.

The presence of free water on the leaves of the plants at the time of inoculation favored the initiation of infection in two ways: the droplets of water helped the conidia or fragments of mycelium to adhere to the leaves, and the presence of free water favored the immediate germination of the conidia or resumption of growth of the fragments.

The 10-hr. period of high moisture after inoculation required for the initiation of infection at 22° C. was slightly longer than the periods found to be necessary by Hemmi and Nojima (1). Whether this difference may be rightly ascribed to differences in the strain of *Helminthosporium oryzae* or to other causes is not known; however, the fact that the optimal temperature for infection was lower than that reported for the Oriental isolates would provide supporting evidence that there were differences between the isolates.

Rain is a factor that is not often considered as one that hinders the development of disease. While the disease may be spread to a limited extent by splashing, it appears that the principal effect of rain is to wash the conidia from the leaves. In the field it has been noted that the seasons most favorable for the development and spread of brown spot were those in which there were heavy dews but little rain.

The optimal temperature for infection by the conidia or mycelial fragments was 20–25° C., a range lower than that reported by Hemmi and Nojima (1) who considered 25–30° C. optimal. It might be mentioned also that the optimal temperatures for growth and conidium formation on agar were lower for this isolate than those reported by workers who used Oriental isolates.

The infectivity of fragments of mycelium is of interest from two points of view. It is possible that fragments of vegetative hyphae or of conidiophores, as well as conidia, may play a rôle in the dissemination of the fungus although conidia generally are considered the entities primarily responsible for dissemination of the fungus in the field. From the point of view of the investigator, the use of mycelial fragments as inoculum for field experiments eliminates the necessity of limiting the testing of isolates of a pathogen to those which sporulate. The use of such fragments also permits production of inoculum in submerged culture, where mycelium can be grown rapidly and conveniently in comparatively large quantities. With large amounts of inoculum on hand, the investigator may run large field tests for varietal susceptibility with an organism of known characteristics instead of depending on natural infection.

## SUMMARY

1. Methods are described for the production of conidial and mycelial inocula for use in greenhouse and field experiments. Mycelial fragments produced in submerged aerated liquid culture proved infective, though less so than conidia.

2. Rice plants could not be predisposed to infection by *Helminthosporium oryzae* by keeping them at high humidity prior to inoculation; however, the presence of free water on the leaves favored the development of infection.

3. A 10-hr. period of high moisture after inoculation was required for the establishment of infection at 22° C. Rain reduced the amount of infection by washing the inoculum from the leaves.

4. The temperature range required for establishment of infection was 20–30° C., and the optimal range was 20–25° C.

5. In the field an evening inoculation resulted in more severe infection than morning or noon inoculation.

6. Rice was susceptible to leaf infection at all stages of development.

7. No varietal resistance to brown spot was demonstrated.

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# THE CAUSAL AGENT OF BACTERIAL ROOT AND STEM DISEASE OF GUAYULE

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Late in 1945, the writer was consulted about the identity of the bacterium which Campbell (5) had shown to cause a root and stem disease of the rubber-bearing plant, guayule (*Parthenium argentatum* A. Gray). Cultures of bacteria isolated from naturally and artifically infected guayule were received from W. A. Campbell in December, 1945. It is the purpose of this communication to describe these cultures and to discuss the taxonomy and nomenclature of this organism.

## METHODS

The purity of all cultures was assured by microscopic examination, and by plating from dilute aqueous suspensions onto yeast agar. From isolated colonies, the cultures were restreaked on yeast agar, and from colonies derived from these second platings, a third streaking was prepared in the same way. All of the second and third plates showed but one type of colony. A single colony was fished from each plate of the third series and shown to be capable of inducing typical symptoms (5) when inoculated into guayule plants. These pure and virulent cultures (referred to hereafter as "original pure cultures") were then run through the determinative tests which are presented below.

In general the methods employed are those given in the Manual of Methods for Pure Culture Study of Bacteria (15), and in the Manual of Dehydrated Culture Media and Reagents (6). When a modification, or a technique not described in either of these publications, was used, appropriate citation or description of the method is given.

Ten isolates and reisolates, and sometimes a larger number, were used in all tests; unless otherwise indicated, all cultures behaved similarly. Except where stated otherwise, cultures were incubated at 28° C.

[1] National Research Council Fellow, 1944–1946, while on leave from the Department of Biology, Brooklyn College, Brooklyn 10, New York. The studies upon which this report is based were conducted at the Hopkins Marine Station of Stanford University, Pacific Grove, California.

[2] The writer acknowledges with thanks the cooperation of Dr. W. A. Campbell, Special Guayule Research Project, Salinas, California, throughout the course of this study. He is greatly indebted to the staff of the Ninth Service Command Medical Laboratory, U. S. Army, Monterey, California, and to that organization's Commanding Officer, Colonel Francis E. Council, M.C., for material aid. To Professor C. B. van Niel, he is very grateful for stimulating discussion, especially concerning systematics.

## DESCRIPTION OF THE PATHOGEN

### *Morphology and Staining Reactions*

The guayule pathogen is rod-shaped. From 18-hour-old yeast-agar or broth cultures, the cells are cylindrical with hemispherical ends, and about 0.7–0.8 by 3–7 μ or sometimes longer. Older cells are shorter and thinner and include, at times, curved, club-shaped, spindle-shaped, and other involution forms. The cells usually occur singly, and sometimes in pairs.

Young cells stain well with gentian violet, fuchsin, and methylene blue. Older cells stain poorly even after long exposure to gentian violet and fuchsin; methylene blue is a somewhat better stain for such old cells. The guayule pathogen is Gram-negative by both the Hucker and the Kopeloff and Beerman modifications of Gram's stain. This statement is based upon examination of cultures, from both sugar-free and sugar-containing liquid and solid media, ranging in age from 12 hours to 3 months. The guayule pathogen is not acid-fast.

The guayule pathogen is actively motile, motility being retained for more than a week in many cultures. By means of the Zettnow (11) and the Gray flagella stains, peritrichous flagellar arrangement can be demonstrated; usually there are 6 to 12 flagella per cell, each about 1.5 to 2 times as long as the bacterium.

Endospores are not formed. Although some of the more lightly staining older cultures contain cells which are superficially spore-like in that the center of the cell remains unstained while the poles and periphery stain, these cells cannot be stained by Dorner's or Ziehl-Neelsen's spore stains, the central regions are not refractile like spores, nor do they survive heating to 85° C. for ten minutes as do true endospores. Inasmuch as stained preparations from young cultures sometimes show polar areas more deeply colored than the centers, the "spore-like" cells may come about as a result of the over-all reduced stainability of the older cells. These central areas do not stain with iodine.

Capsules are seen about the cells in many preparations, notably in smears stained by Gray's flagella stain. By means of Hiss's capsule stain, narrow capsules could be demonstrated surrounding cells from 7-day-old dextrose-yeast-agar cultures.

### *Cultural Characteristics*

*Colonies on yeast agar and on beef-peptone agar.*—The yeast agar was made with 1 per cent Bacto yeast extract and 2 per cent Bacto agar, and its pH was 6.8. The beef-peptone agar contained 0.3 per cent Bacto beef extract, 0.5 per cent Bacto peptone, and 2 per cent agar; its pH was 6.8. Surface colonies are flat, greyish, iridescent, dry and not glistening, almost transparent, approximately round, with amoeboid edges. The surfaces of most colonies appear wrinkled to the naked eye; at magnifications of 50–100 ×, shallow, irregular furrows can be seen, and the centers and peripheries appear a little raised. The texture is amorphous or finely granular

at 150×. The growth is readily suspended in water and is not viscid. Well-isolated surface colonies are ordinarily under 1 cm. in diameter. Subsurface colonies are lenticular, opaque, and usually about 1 mm. long.

*Colonies on yeast-dextrose agar (yeast agar plus 2 per cent dextrose).*—Surface colonies are slightly raised, with light tan or cream centers and practically colorless peripheries, round with amoeboid edges, translucent, not mucoid or viscid. The surface is wrinkled or stippled, and the size usually under 1 cm.

*Colonies on yeast-dextrose-$CaCO_3$ agar—"YDC" (yeast-dextrose agar plus 2 per cent precipitated chalk).*—Colonies on this medium are generally similar to those on yeast-dextrose agar. The $CaCO_3$ is decomposed, more so at times than others, resulting in a cleared space beneath the colony not ordinarily extending beyond the edge. At times, a layer of redeposited $CaCO_3$ can be seen at the margin of the colony.

By plating the original pure cultures on this medium, there could be obtained from each culture two colony types which differ in the rate and extent of clearing of the $CaCO_3$. The more extensively decomposing sort (type A) is very mucoid on this medium, although the slime seems to dry out after a few days. In addition, colonies of type A appear somewhat tan by contrast with the less extensively decomposing type B, because of the complete dissolution of the chalk beneath the type A colony with the result that the brown color of the medium is visible through the translucent growth. Types A and B differ in other characteristics, notably in reaction on eosin-methylene blue agar, in milk, and in broth, as described in the appropriate places below. This phenomenon probably represents a transition somewhere in the series Rough→Smooth→Mucoid, with a concurrent change in the mode or rate of metabolism of sugar.

Unless otherwise specified, the descriptive matter presented herein refers to the original pure cultures, which were probably entirely in the type B condition.

*Eosin–methylene blue agar—"EMB."*—Two types of reaction are observed on this Bacto medium containing both lactose and sucrose: the more common is formation of colonies similar in size and shape to those seen on yeast-dextrose agar, although sometimes more moist and spreading; the color is pink, often with blue or violet centers, sometimes becoming entirely blue or violet. The other reaction is production of dark violet colonies which have a green-metallic sheen by reflected light, and are often exceedingly mucoid. Some of the original pure cultures yielded a large proportion of greening colonies; in others they were rare or absent. From papillae on nongreening colonies and on yeast-agar-slant growth, or by repeated plating of each of the original pure cultures on EMB, there could be isolated more or less pure strains of the greening variety. Both the greening and the nongreening sorts were unstable, and reverted to mixtures; the nongreening type seemed the more stable of the two. At the same time, the two colony types on YDC, discussed in the paragraphs above, had been

found. Cultivation of the EMB varieties on YDC, and of the YDC types on EMB, showed that they are probably duplicates; the greening variety corresponding to type A, and the nongreening sort to type B. Both EMB varieties proved to be virulent to guayule and neither of them fermented filter-sterilized lactose any more quickly than did the original pure cultures. However, type A did produce an acid reaction in autoclaved litmus milk, in contrast to the alkaline peptonization brought about by the type B cultures.

*Growth on yeast-agar slants.*—Flat, practically colorless, not spreading, with irregular margins, iridescent, not viscid. At times, and especially on moister slants, the growth becomes heavier, whiter, more spreading and wet-looking. This may be related to the changes noted for colonies on YDC.

*Growth on yeast-dextrose-agar slants.*—Wet-looking, whitish, slightly raised, abundant, spreading, iridescent, not mucoid or viscid.

*Growth on yeast-dextrose-$CaCO_3$-agar slants.*—Flat, colorless to cream, with slightly irregular edges, dry and not glistening. This is probably the type B growth; nothing like type A was seen on these slant cultures, but no special effort was made to secure this modification.

*Yeast or beef-peptone broth.*—Definitely turbid within 12 hours. On further incubation of type B cultures, the turbidity increases, a finely-granular, very thin, easily dislodged pellicle forms, and a smooth or finely granular sediment is deposited. Type A cultures have heavier sediments and are less turbid than type B.

*Yeast-dextrose broth.*—Heavy turbidity, abundant viscid or slightly granular sediment, white ring, easily dislodged granular pellicle. The type A cultures have generally a more abundant and more granular sediment and are less turbid than type B. In the better-buffered dextrose-peptone broth used for the Voges-Proskauer tests, growth is somewhat heavier and these differences between types A and B are accentuated.

*Action on autoclaved litmus milk (Bacto), on autoclaved brom cresol purple milk, and on autoclaved skim milk.*—Peptonization starts in the type B cultures after about a week and continues until the entire tube is cleared—meanwhile, the indicator shows an alkaline reaction. The type A cultures usually make an acid curd after 10 to 14 days, the litmus is reduced, and no peptonization occurs.

*Potato cylinders.*—No growth, or only a trace of growth, on the surface of the potato. Moderate growth in the liquid below the potato.

*Relationship to oxygen.*—In thioglycollate broth (Bacto) and in yeast-agar shake cultures, heavy growth occurs only in the upper 1 to 2 mm., with no growth below, showing a distinct preference for aerobic conditions. In yeast-dextrose-agar shake cultures, there is growth throughout the tube, and the agar is blown apart by gas formation, illustrating the organism's ability of growing anaerobically by means of its fermentative metabolism. The latter capacity is revealed also by growth and the formation of acid and gas in a synthetic dextrose medium (17), within the closed arm of Smith fermentation tubes.

*Catalase.*—Catalase in cultures on various media can be shown by the evolution of gas when $H_2O_2$ is added.

*Chromogenesis.*—Other than occasional light tan or cream growth on some agar media, there has been observed no distinct coloration of the cell mass or medium.

### *Carbon and Nitrogen Metabolism*

*Minimal nutritive requirements.*—The guayule pathogen grows moderately well from small inocula in a synthetic medium (17) containing $NH_4Cl$, $MgSO_4$, phosphate buffer, "trace" elements, and separately sterilized, purified dextrose. More rapid growth and a somewhat larger crop results from the addition of 0.5 per cent "vitamin-free" casein hydrolyzate. At least part of this apparent stimulation is due to the buffering effect of the amino acids or their degradation products which maintain the pH at a level suitable for continued growth; the limiting pH of 4.2 was reached in a few days in the $NH_4Cl$-dextrose medium, whereas, even after 20 days' incubation, the pH in the casein hydrolyzate medium was only about 5.0. A mixture of known vitamins has no detectable effect upon growth.

*Utilization of sodium citrate as sole carbon source.*—All cultures grow moderately well on Bacto Simmons citrate agar and the pH-indicator shows an alkaline reaction, thus indicating that the guayule bacterium can use citrate as sole carbon source.

*Action on sugars.*—The ability to dissimilate sugars was determined in a medium containing 0.3 per cent beef extract, 0.5 per cent peptone, Andrade's indicator; pH 6.7. Sugars were sterilized by passage through Seitz filters and were used in final concentrations of 1 per cent in Durham tubes. Acid and gas (generally under 10 per cent) are formed within 48 hours from arabinose, dextrose, galactose, levulose, maltose, raffinose, salicin, sucrose. Somewhat slower production of acid and gas takes place from cellobiose (7 days), lactose (14 to 20 days), rhamnose (7 days), xylose (2 to 4 days). Acid, but no detectable gas, is formed slowly from inositol. No acid or gas is produced after a month's incubation from dextrin, inulin, mannitol, sorbitol, trehalose, and sugar-free base. In many cases, an originally acid reaction became alkaline after longer incubation.

*Methyl red and Voges-Proskauer tests (Bacto M.R.–V.P. medium).*—After 48 hours there is a negative methyl red test, which remains negative for a month. The Voges-Proskauer test is weakly positive or questionable for the first week, later becoming more distinctly positive. Acetyl-methyl-carbinol was sought by adding a few drops of $FeCl_3$ solution and 5 ml. of 10 per cent KOH to 5 ml. of culture; the tests were incubated at 37° C. for several hours before the final readings were made.

*Action on starch.*—The starch agar had 0.5 per cent yeast extract, 0.2 per cent soluble starch, and 2 per cent agar; its pH was 6.8. Starch broth was the same, minus agar. Good growth on the starch agar, but no action on the starch was detectable by flooding the plates with Lugol's iodine solution after 3, 7, or 20 days. Lack of diastatic power was verified in the liquid

medium, in which there is good growth, but a negative iodine test, and no acid or reducing sugar, after 2, 7, or 12 days.

*Action on sodium ammonium pectate.*—A pectate medium was prepared by dissolving 3 g. of sodium ammonium pectate (No. 24 of California Fruit Growers Exchange, Ontario, California) in 100 ml. distilled water to which had previously been added 0.9 ml. normal NaOH, 0.6 ml. 10 per cent $CaCl_2 \cdot 2H_2O$, and brom thymol blue as indicator. After complete solution on a water bath, the pH was adjusted to 7.3, the medium tubed and autoclaved, after which the reaction was pH 6.4. A yeast-pectate medium was prepared in the same way by adding 1 per cent yeast extract to the above. After the media had gelled, they were inoculated by stabbing. The guayule pathogen grows well within 24 hours in both media, and with particular luxuriance in yeast-pectate. After 2 days, the growth occupies a carrot-shaped space reaching to the bottom of the tube, and both media are liquefied, ranging, with the different isolates, from a few millimeters at the surface to practically complete liquefaction. After a week or 10 days, all cultures are completely liquid and the reaction, which at first is acid, becomes neutral.

*Pectolytic action on slices of raw carrot.*—All cultures produce an active, soft rot of raw carrot slices within 2 days.

*Action on guayule resin.*—On Allen, Naghski, and Hoover's (1) resin agar and on resin-yeast agar there is good growth, but no decomposition of the guayule resin on either of these media after 7 days.[3] Resin-decomposing bacteria tested at the same time brought about distinct clearing of the resin about the colonies.

*Action on gelatin.*—The medium contained 0.3 per cent beef extract, 0.5 per cent peptone, and 12 per cent Bacto gelatin; pH was 6.6. A second batch contained 1 per cent yeast extract, 12 per cent Bacto gelatin; pH was 6.0. In both media, there is a funnel-shaped liquid zone at the end of 2 days at 22° C.; within a week or 10 days, the entire tube is liquefied.

*Indole production.*—On 1 per cent Bacto tryptone at pH 7.0 indole is formed in 2 days as shown with Kovács' reagent (2).

*Hydrogen sulphide production.*—Questionable in Bacto lead acetate agar. Slight browning, appearing in 3 days but not progressing much thereafter, of lead acetate paper suspended over cultures in 1 per cent tryptone, or on yeast-agar slants.

*Reduction of nitrates.*—On a medium of 0.3 per cent beef extract, 0.5 per cent peptone, 0.1 per cent $KNO_3$, 1.2 per cent agar, at pH 6.8, a strongly positive test for nitrites is obtained after 2 days using the sulfanilic acid and α-naphthylamine reagents.

*Tyrosinase production.*—Tyrosine and control media (16) were modified by omitting the agar and adding 0.1 per cent glutamic acid; pH was 7.8. Abundant growth, but no definite coloration of either medium after 30 days, indicating the absence of tyrosinase.

[3] The cooperation of Dr. Paul J. Allen in performing these tests is greatly appreciated.

ATTEMPTS TO INFECT GUAYULE WITH OTHER SOFT-ROT BACTERIA

After the determinative tests made it appear likely that the guayule pathogen was a species of *Erwinia*, several attempts were made to infect guayule plants with virulent soft-rot bacteria of that genus isolated from other hosts. The following eleven isolates[4] were studied: five isolates of *Erwinia carotovora*, one isolated from cabbage and sent by E. H. Garrard, the others isolated from yellow calla, carrot, celery, and larkspur and received from P. A. Ark; two isolates of *E. aroideae*, one sent by E. H. Garrard, and the other isolated from tobacco stems and received from W. J. Dowson; *E. oleraceae*, isolated from cauliflower and sent by E. H. Garrard; *E. rhapontici*, isolated from rhubarb and sent by W. J. Dowson; *E. solanisapra* and *E. atroseptica*, both isolated from potato and sent by E. H. Garrard.

In conducting these tests, greenhouse-grown guayule seedlings were used; these had been cultivated in sterilized soil in 6-inch pots. Each isolate was inoculated into 4 to 9 plants. Inoculations were made approximately an inch below the soil surface in the following manner: The soil was removed from the root crown and upper tap root, and a series of small punctures made in the bark with a sharp-pointed scalpel. Bacteria, grown on nutrient-dextrose agar, were applied to the wounds, the soil replaced, and the plants watered immediately. The checks were treated with sterile agar. At least one virulent strain of the guayule pathogen was included in each series as a positive control. After inoculating, the plants were watered every 2 or 3 hours for the first two days, and then 3 times a day thereafter.

In the first series of tests, with Ark's isolates of *Erwinia carotovora*, inoculated plants were kept in tanks at nine different soil temperatures between 13° C. and 37° C., in an effort to provide optimal conditions for infection by these organisms (5). In later tests, with the other isolates of *Erwinia*, plants were held at the usual greenhouse temperatures, since these temperatures had, by that time, been shown to be well within the range for consistently successful infection of guayule by the guayule pathogen.

None of the soft-rot bacteria from hosts other than guayule, when inoculated into guayule, was able to cause the disease under consideration.[5] The check plants, to which only sterile agar had been applied, remained uniformly healthy. The positive controls, inoculated with the guayule pathogen, quickly came down with the typical disease (See figure 1). Since the soft-rot cultures that were used are fairly representative of the entire group, it is probably correct to consider the guayule bacterium unique in the ability of causing the root and stem disease of guayule.

[4] The cultures are listed by the names under which they were received. The writer wishes to express his thanks to the donors of these cultures.

[5] Dr. W. A. Campbell, who very kindly conducted and interpreted these tests, reported that a total of three plants, out of the eight that had been inoculated with *Erwinia atroseptica* and Dowson's *E. aroideae*, "had a soft-rot of the root, which could be caused by drowning (13), but none had typical symptoms of guayule rot; *i.e.*, resin flow on lower stem and progress of rot into stem."

TAXONOMY AND NOMENCLATURE

The question of assigning a name to the guayule pathogen is a perplexing one. In accordance with current American usage, as in Bergey's Manual of Determinative Bacteriology, this organism undoubtedly belongs, with other peritrichous, nonsporulating, pectolytic bacteria, in the soft-rot section of the genus *Erwinia*. By Dowson's (7) classification, which has merit, it would be considered a species of *Bacterium*. In other, less generally accepted, and, by certain standards, unjustified, systems, it would be placed in *Paracolobactrum* (4) or *Pectobacterium* (20).

While disposition as to genus is relatively easy, considerably more difficulty attends allocation of this organism to a species. Opinion on the

FIG. 1. Guayule plants in constant temperature tanks at 27° C. (right half) and 30° C. (left half). The plants in the first (bottom) row were inoculated with strain 263C of the guayule pathogen and had typical advanced symptoms of the root and stem disease; the controls (second row) were wounded but treated only with sterile agar; the plants in the third and fourth (top) rows were inoculated with the cultures of *Erwinia carotovora* originally isolated from carrot, yellow calla, larkspur, and celery by P. A. Ark. The control plants and those inoculated with the *E. carotovora* strains from hosts other than guayule were healthy and the inoculation wounds had healed. Inoculated Jan. 31, 1946, and photographed by W. A. Campbell Feb. 21, 1946.

speciation of soft-rot bacteria ranges all the way from realistic arrangements wherein all strains of the soft-rot *Erwinia* are lumped into one or two flexible, but variously described, species, to schemes in which a large number of so-called species are separated on the bases of more-or-less justifiable pathological, serological, biochemical, and cultural distinctions. (See references 3, 7, 8, 10, and 20, for discussions of this subject.)

Naming a bacterium of plant disease origin often brings to the fore the conflict between the "utilitarian" and the "scientific" aspects of microbial taxonomy (19). The practicing plant pathologist is primarily concerned

with the disease-producing abilities of the bacterium and, in so far as possible, prefers nomenclature which in some way indicates this pathogenicity. The fulfillment of this perfectly reasonable, but what must be recognized as "utilitarian" aim has often led to considerable synonymy when the microorganisms are considered bacteriologically apart from their pathological properties. Much confusion and polemic would be avoided if both needs were satisfied—that is, if the nomenclature reflected "scientific" bacteriological relationships as well as "utilitarian" phytopathogenic abilities. The guayule pathogen is a case in point: as a bacteriological unit, it differs not at all from the range of strains which constitute *Erwinia carotovora;* nor, for that matter, is it unlike the group of saprophytic slow-lactose-fermenting "aberrant coliforms" (18). Granted, the guayule pathogen does have cultural properties which, according to various published descriptions, might differentiate it from the average strain of *E. carotovora* (for example, indol production, peptonization of milk, failure to grow on autoclaved potato); however, these distinctions, which in some quarters would be used to separate "species," are well within the limits of variability of the coliform species.

On the other hand, considered from the pathologist's viewpoint, the guayule bacterium does attack guayule, whereas that plant is not infected by virulent soft-rot isolates from other hosts. Although it is debatable whether such host specificity is sufficient justification for erecting a bacterial species, this characteristic is of prime practical importance to the plant pathologist. Incorporation of the guayule pathogen into one of the present *Erwinia* species, without in some way indicating its special ability of infecting guayule, would, therefore, be unwise.

The dilemma can be met by naming the guayule pathogen as a special form or pathogenic variety of *Erwinia carotovora.* There is ample precedent for such action (9, 12, 14). The term "variety" has had two uses in the classification of phytopathogenic bacteria; firstly, to distinguish culturally different organisms which cause indistinguishable diseases on the same host, and secondly, to differentiate organisms which are considered culturally identical, but capable of infecting different hosts. It is suggested that "variety" be restricted to labeling cultural differences, and that the term "*forma specialis*" be used, in the sense recommended in the International Rules of Botanical Nomenclature (See 9), for distinguishing "within the species special forms characterized by their adaptation to different hosts."

In accordance with the foregoing discussion, the name *Erwinia carotovora* f. sp. *parthenii*, f. sp. nov., is suggested for the above-described guayule pathogen. (Synonym: *Bacterium carotovorum* f. sp. *parthenii.*)

## SUMMARY

The bacterium which causes a root and stem disease of the rubber-bearing plant, guayule (*Parthenium argentatum*), is described and its sys-

tematic position is discussed. The name *Erwinia carotovora* f. sp. *parthenii*, f. sp. nov., is suggested for this organism.

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# A WILT-INDUCING TOXIC SUBSTANCE FROM CROWN-GALL BACTERIA[1]

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## INTRODUCTION

Investigators of crown gall have employed various techniques for introducing into the plant certain preparations from cultures of the causal bacterium. The crown-gall studies have been reviewed by Riker and Berge (32), Levine (21), and Riker (31). Extracts of whole cultures, of cells, and of culture filtrates have been applied directly, or mixed with a carrier such as lanolin, to the plant with or without wounding (6, 22). Some samples were injected (5). A technique common with investigations on wilt-inducing microorganisms (but apparently not employed previously on crown-gall bacterial filtrates) is to immerse the cut end of a plant shoot in a solution of the culture filtrate. Tomato cuttings were tested in this manner. After they had stood 48 hours in a cell-free filtrate from a crown-gall bacterial culture, the leaflets showed a severe wilting followed by necrosis. Since some metabolite presumably caused these effects, it was sought by fractionation.

The symptoms induced by these filtrates were similar to some of those reported for wilt-inducing microorganisms. The extensive literature about wilting has been reviewed most recently by Brown (7), Harris (13), and Gottlieb (11).

Various theories have arisen concerning the wilting mechanism involved when the wilting symptoms could not be ascribed to tissue disintegration. Mechanical plugging of the plants' conducting vessels by bacteria and their products (36) or by fungal mycelia (35) was early cited as the cause of wilt.

Later investigators gave other explanations. Tochinai (37) suggested that the microorganism growing in the vascular system formed gas emboli, which blocked the transpiration stream. Hutchinson (20) considered that wilting was caused by toxic substances elaborated by the wilt organisms. This toxic-filtrate theory has received considerable support, *e.g.*, recently from Wellman (38), Gottlieb (10, 11), Clauson-Kaas, Plattner, and Gäumann (8), and Plattner and Clauson-Kaas (28, 29).

Chemical studies of such toxic filtrates are relatively few. Several workers have obtained alcohol precipitates that were toxic. Certain of these were thermostable (2, 27) while others were thermolabile (3, 12, 39). The character of such precipitates generally was not investigated. Luz (25) attributed the wilting activity of a Fusarium filtrate to ammonia, which also

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was reported by others who did not find a sufficient amount to account for the toxic effects (9).

The association of amino compounds with the main toxic fractions of fungus filtrates was suggested by Liesau (23). Various amines, amino acids, and other nitrogenous compounds were tested by Schaffnit and Lüdtke (34), Ahmet (1), and Lüdtke and Achmed (24). Aqueous solutions (0.5 per cent concentration) of certain of these, *e.g.*, creatine, putrescine dihydrochloride, colamine, and others, had wilting activity similar to that induced by toxic preparations from Fusarium cultures. Although these workers obtained concentrates of the toxic material, isolation and characterization of any compounds were not reported. More recently Clauson-Kaas, Plattner, and Gäumann (8) reported the isolation of a wilt-inducing metabolic product from old cultures of *Fusarium lycopersici* Sacc. This substance was named lyco-marasmin (28, 29) and was a low molecular weight peptide having an empirical formula of $C_9H_{15}O_7N_3$. Upon hydrolysis it yielded ammonia, glycine, aspartic acid, and a product that appeared to be pyruvic acid. A trace of ferric chloride greatly enhanced the activity of this compound.

The purpose of the present paper is to describe a method of measuring the toxic substance produced in cultures of the crown-gall organism and to describe its concentration and chemical properties. An abstract of this work has already appeared (17).

## EXPERIMENTAL WORK

### *Materials and Methods*

*Cultures and medium.*—Two strains of *Phytomonas tumefaciens* (Smith and Townsend) Bergey *et al.* were employed. Both the virulent strain, A6 (40), and the attenuated daughter strain, A6–6 (16), were progenies of single cells. The activity of these cultures was checked at intervals by inoculations into tomato plants. In all tests the virulent strain induced galls while the attenuated strain produced only rudimentary or no galls.

The sucrose, urea, mineral-salts medium and the methods employed in growing the bacteria were the same as those described earlier (26), except in some cases the incubation time was longer. All cultures were incubated in liter flasks at approximately 26° C. Aeration was provided by placing the flasks in a shaker having a 5-inch reciprocating stroke and running at 54 strokes per minute. Each flask contained 200 ml. of medium and was provided with a 6-inch extension neck to prevent wetting of the cover. Instead of cotton plugs, a double layer of 6-ply, oil-treated air filter tissue was used to prevent contamination and to provide increased and uniform entrance of air.

*Analytical methods.*—Reducing sugar was determined (after hydrolysis in the case of sucrose or polysaccharide) according to the method of Shaffer and Somogyi (33) (Reagent 50 with 5 g. of potassium iodide per liter). Determinations of pH were made with the Beckman pH meter. Osmotic

pressure was measured by the cryoscopic method according to Harris and Gortner (14).

*Plant Assay*

To aid the chemical study a suitable method was developed for estimating the potency of filtrates, extracts, and concentrates.

Neither filtering nor autoclaving (solution near neutrality) appreciably influenced the wilt-inducing activity of filtrates. Thus, filtrates free from viable cells were prepared from liquid cultures of the crown-gall organism (a) by filtering through a Seitz or a sintered-glass filter or (b) by filtering through a layer of asbestos and Hyflo Super-Cel followed by autoclaving at 15 lb. steam pressure for 20 minutes.

Bonny Best tomato plants for the assay were grown in the greenhouse. In 10 to 14 days after seeding, they were well spaced as they were transplanted into a flat of composted soil containing approximately 25 per cent sand. The plants were used after 2 or 3 weeks when they were 12 to 18 inches in height. Each plant was selected for uniformity of size and leaf area; the upper stem bearing 2 or 3 well-developed leaves was cut under water and left standing in tap water until transferred into the test solution.

The tests were made in a small basement room without windows. Light from fluorescent lamps (three 30-watt bulbs at approximately 30 inches) shone on the cuttings for 18 hours each day. The temperature was 25° C. ($\pm$ 2°) and the relative humidity was 70 per cent ($\pm$ 10 per cent).

The cuttings remained in tap water about an hour before they were transferred into graduated 10-ml. vials containing the test solutions. Graduated 25-ml. test tubes also were employed satisfactorily. Either the volume of the test solution or the weight of the solution and vial was noted before transferring the cuttings. After 46 to 50 hours the amount of liquid absorbed and the degree of wilting were recorded. In some tests the loss in weight of solution from the vials (*i.e.*, the amount taken up by the cutting) was obtained to the nearest 0.01 g., while in others the loss in volume was read to the nearest 0.1 ml. Correction was made for the amount of moisture lost by evaporation from control vials containing no plant cuttings.

The progressive symptoms began with tips of the leaflets becoming wilted and tending to cling to any object touched. Later, margins of the leaflets became curled or rolled. The injury gradually spread to the remainder of the leaflets, while the parts first affected became shriveled and necrotic (Fig. 1). In severe cases the entire leaflets became shriveled, dried, and dead. Successive stages are drawn in figure 2. The stems and main petioles, however, remained turgid although epinasty was apparent in some cuttings. Small, irregular, dark, necrotic areas sometimes appeared on the leaves. The portions of the stem below the surface of the liquid in some cases became softened.

In this paper, such symptoms are referred to broadly as "toxic effects," "injury," or "wilting." "Wilting" has been used frequently as synony-

mous with "toxic effect" by several authors to describe symptoms induced by filtrates from various organisms (19, 34, 39).

The degree of injury or "toxic index" was recorded as 0 to 5 according to severity, as shown in figure 2. Intermediates were sometimes recorded as 1.5, 2.5, . . . .

FIG. 1. Left: tomato cutting in filtrate from crown-gall bacteria showing a severe wilting and necrosis of the leaflets (toxic index of 4) after 48 hours at 27° C. and at a relative humidity of 80 per cent. Right: tomato cutting in control medium showing no wilting (toxic index of 0).

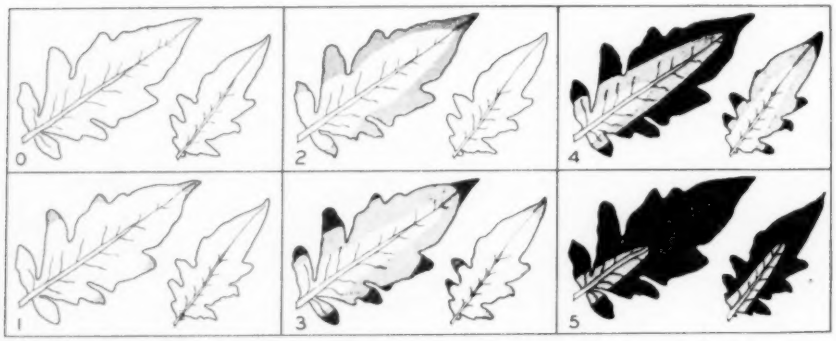


FIG. 2. Method of recording toxic indexes. The small leaf represents an upper and younger leaflet, and the large one represents an older and lower leaflet. Shaded areas represent wilted portions; blackened areas represent necrotic and collapsed portions. 0, no apparent symptoms. 1, wilting is just apparent on the tips of the lower and larger leaflets. 2, edges, as well as the tips of the larger leaflets, are wilted and curled. 3, tips, edges, and part of the inner area of the lower leaflets are severely wilted and curled, and the tips are necrotic; wilting is beginning on the upper leaflets. 4, lower leaflets are curled, necrotic, and collapsed; edges of upper leaflets are wilted and curled. 5, all leaflets are collapsed and necrotic; some may be shriveled and dried; stems are turgid.

*Factors affecting the assay.*—Two tests on the effect of temperature suggested that the toxic index increased with higher temperatures. Solution intake was greater at higher temperatures with unfermented medium but not with the filtrates. This corresponds with Haymaker's (15) report that tomato plants, whose cut ends were immersed in extracts from *Fusarium lycopersici*, showed symptoms more quickly at higher temperatures and lower humidities.

The effect of pH was studied in 4 trials. The toxic index of plants in filtrates from either the attenuated or the virulent organism was approximately the same at pH levels of 3.0, 5.0, or 7.0. Therefore, in subsequent experiments, some samples were adjusted to pH 5.0, but most were adjusted to pH 3.0 before assay in order to retard growth of contaminating microorganisms. Cuttings in control medium (sucrose, urea, mineral-salts) adjusted to pH 3.0 or 5.0 did not wilt.

In relation to time, readings were taken on many samples at both 24 and 48 hours. A representative comparison of these readings is given in table 1.

TABLE 1.—*Effect of time on solution intake and toxic index of cuttings in filtrates from crown-gall bacteria*[a]

| Dilution | Solution intake after | | Toxic index after | |
|---|---|---|---|---|
| | 24 hours | 48 hours | 24 hours | 48 hours |
| | *Ml.* | *Ml.* | | |
| 1.25 | 2.6 | 3.1 | 2.0 | 3.5 |
| 2.5 | 4.0 | 5.1 | 1.1 | 3.7 |
| 5.0 | 6.3 | 8.7 | 0.6 | 2.3 |
| 10 | 5.0 | 7.0 | 0.0 | 2.2 |
| 20 | 6.3 | 9.1 | 0.0 | 0.8 |
| 40 | 6.6 | 10.1 | 0.0 | 0.5 |
| Unfermented medium | 4.3 | 6.0 | 0.0 | 0.0 |

[a] Samples were adjusted to pH 5.0. Each figure is the average of 10 cuttings.

Both severity of injury and solution intake increased with time. After 24 hours, cuttings in the more dilute solutions showed no or only slight symptoms, whereas after 48 hours these were mildly or severely injured. Therefore, the 48-hour period seemed preferable and was used in subsequent tests.

For comparison, the unfermented medium was tested and had no toxicity (Table 1). A greater volume of this solution was taken in than with the slightly diluted filtrate, but less than with the more diluted sample. The lower intake of unfermented medium probably is related to its higher content of sucrose and salts. Intake of tap water or distilled water always was greater than that of either the filtrate or unfermented medium. Osmotic pressure (14) could not have been the determining factor in these solution intakes as the unfermented medium and filtrate measured 2.67 and 1.06 atmospheres, respectively. The osmotic pressure of pure distilled water is, of course, 0.

*Reproducibility of assay values.*—Repeated determinations on a given sample were made. Since filtering and autoclaving did not reduce activity, an 8-day-old culture of the virulent organism was filtered through a mat of Hyflo Super-Cel and asbestos; the filtrate was divided into several 100-ml. lots and was autoclaved for 20 minutes at 15 lb. pressure. During 3 weeks samples were assayed 4 separate times, and each experiment was set up at 6 dilutions with 10 plants at each dilution. Average values for the volume of the solution taken up and the toxic indexes recorded after 48 hours are given in figure 3, and the various relationships are indicated. The plants in the stronger solutions showed a greater degree of wilting for the amounts of liquid taken in than the plants in weaker solutions.

The data from each series were analyzed statistically[2] (Table 2). The differences between dilutions for toxic index and for solution intake were

TABLE 2.—*Analysis of variance of the data shown graphically in figure 2*

| Source of variance | Degrees of freedom | Mean squares of | |
|---|---|---|---|
| | | Solution intake | Toxic index |
| Dilution | 5 | 342.01[a] | 63.24[a] |
| Linear component | 1 | 1,675.40[a] | 311.56[a] |
| Series | 3 | 4.47 | 7.62[a] |
| Error (1)[b] | 15 | 7.56 | 0.88 |
| Error (2)[c] | 215 | 2.69 | 0.32 |

[a] These values exceed the 1 per cent level of significance.
[b] Error (1) is the interaction of dilution and series and was used to test the significance of dilution and series.
[c] Error (2) is the random variation within dilutions within series.

highly significant. Although an analysis of co-variance indicated a straight-line relationship between solution intake and toxic index at the respective dilutions, no close relation existed between toxic index and solution intake within a given dilution (*i.e.*, actual readings in a given trial hit above and below the line). An analysis of variance, considering each series as one replicate, indicated highly significant $F$ values between dilutions for both toxicity and solution intake. The differences between series were not significant for solution intake but were highly significant for toxic index. This indicated that the method of estimating toxic index (visual judgment) was less precise than that of determining solution intake. Also plants may be more sensitive at one time than another.

The product of the toxic index and dilution when divided by the solution intake yielded a value that was fairly constant at several dilutions. This value was considered as the number of "toxic units" per milliliter in the original solution. Thus, when assayed at a dilution of 1 to 10, a solution that induced a toxic index of 2.2 and a water intake of 10 ml. would have 2.2 toxic units per milliliter; a concentrate that induced a toxic index of 5 and a water intake of 0.5 ml. at a dilution of 1 g. per 100 ml. would contain 1000

[2] The authors wish to thank Professor J. H. Torrie for assistance in the statistical analyses.

toxic units per gram. Thus, a toxic unit is the amount of substance contained in 1 ml. of a solution that caused a toxic index of 1.

This method of calculation was applied to the data from which those given in figure 3 were derived, and the toxic unit values (Table 3) were in fair agreement over the concentration range tested. The precision of the

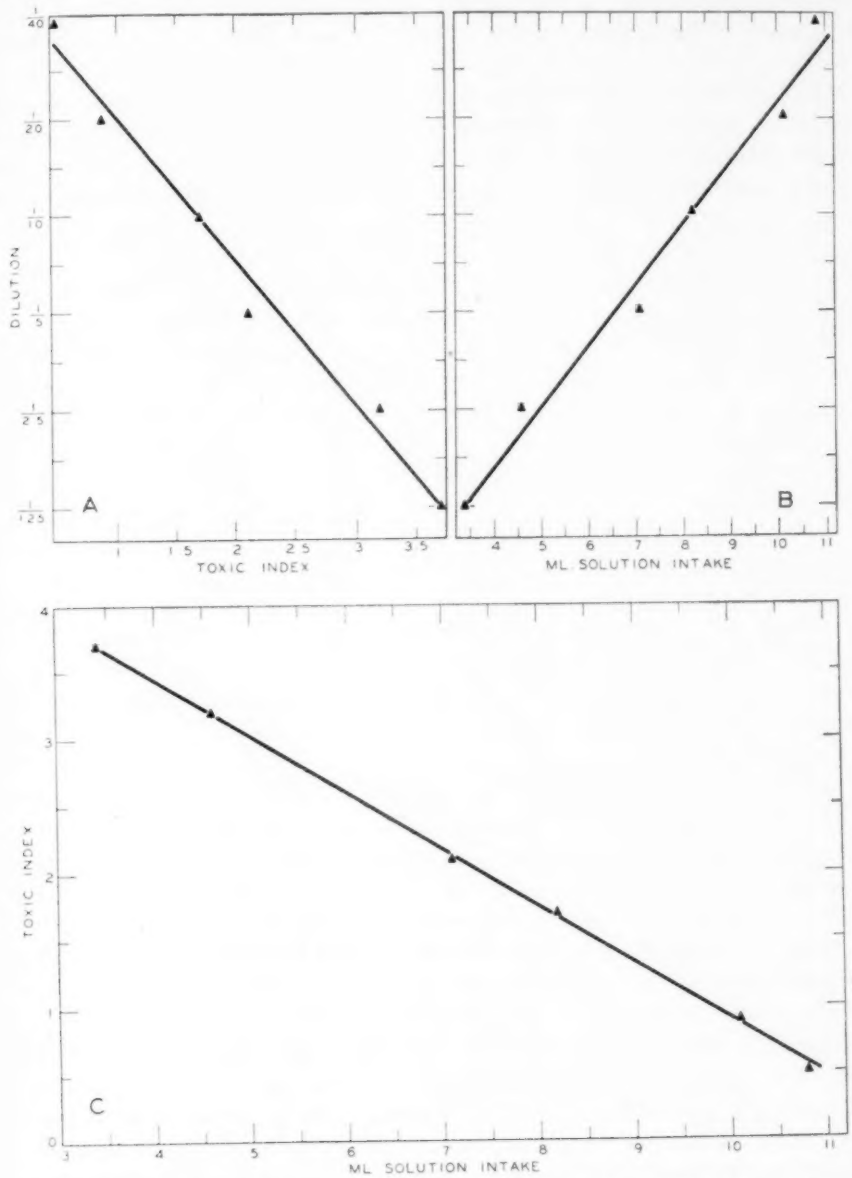


FIG. 3. Relationships of sample dilution, toxic index, and solution intake of tomato cuttings after 48 hours in a filtrate from crown-gall bacteria at 27° C. and 70 per cent relative humidity. Each point is the average of 40 cuttings. A, toxic index and dilutions; B, solution intake and dilution; C, toxic index and solution intake.

assay thus appeared such that the method could be used for a semi-quantitative test. The largest variation (excluding the 0 value) between 0.8 and 3.1 was almost fourfold. Larger variations could be expected with samples replicated with fewer than 10 cuttings. Although control of all other factors influencing the plant assay, such as uniformity of size and leaf area of the test cuttings, was limited, thereby making small differences between toxic unit values difficult to interpret, the method was a useful tool in the chemical investigation.

*Specificity tests.*—The wilting effects described were not specific for filtrates of virulent crown-gall bacteria, since filtrates from the attenuated crown-gall organism (A6–6) also induced similar symptoms. In preliminary tests, few differences, if any, were noted between the filtrate potencies

TABLE 3.—*Toxic units in a fermented eight-day-old culture of virulent crown-gall bacteria*[a]

| Dilution | Series number | | | | Average |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| | *Units per ml.* | *Units per ml.* | *Units per ml.* | *Units per ml.* | *Units per ml.* |
| 1.25 | 1.4 | 1.2 | 1.7 | 1.3 | 1.4 |
| 2.5 | 1.8 | 1.6 | 1.7 | 1.6 | 1.7 |
| 5.0 | 1.3 | 1.1 | 2.0 | 1.4 | 1.7 |
| 10 | 3.1 | 1.3 | 2.2 | 1.9 | 2.2 |
| 20 | 1.8 | 0.8 | 2.3 | 1.9 | 1.7 |
| 40 | 2.0 | 0.0 | 3.0 | 2.4 | 1.8 |
| Average | 1.9 | 1.0 | 2.3 | 1.7 | 1.7 |

[a] Calculated from the data shown graphically in figure 2. Each figure represents the average of 10 cuttings.

of the virulent and the attenuated organisms. The details are omitted for the sake of brevity.

The filtrate from crown-gall bacteria induced wilting of cuttings from other plant species, *e.g.*, sunflower, marigold, and pea. This lack of specificity has been noted also for filtrates from various species of ***Fusarium*** and other microorganisms, which were harmful to a wide variety of plants (2, 4, 12, 34).

Other types of wilting were encountered at various times. Stems of cuttings placed in 0.4 per cent egg albumin suspension wilted in a few hours; wilting of the leaflets followed. Inorganic salts, such as sodium chloride and ammonium sulphate, were tolerated in small concentrations, but in larger concentrations they induced a collapse of the stems that was followed by wilting of the leaflets. Concentrations of sodium chloride less than 0.75 per cent did not induce wilting. However, 0.75 to 1.0 per cent of the salt caused symptoms in some, but not all, of the plants tested, and 1.5 per cent or a higher concentration was very harmful. Plants tolerated concentrations of ammonium sulphate of 0.5 per cent or less, but concentrations of 1 per cent or higher induced similar symptoms.

On several occasions after 70 to 80 cuttings were prepared for an assay, it was noted that both leaflets and stems of 2 to 5 of these sometimes became mildly flaccid, even while standing in tap water. However, these soon recovered when allowed to remain in tap water, and they then appeared normal for several days. This wilting was probably due to the interference with the transpiration stream upon severance of the stem.

*Recovery after wilting.*—Hursh (19) discussed the importance of testing whether cuttings wilted in the so-called toxic filtrates would recover when a little of the stem was clipped off and the remaining portion was transferred to fresh water. This test was applied to cuttings in filtrates from crown-gall bacteria. After 24 hours in the filtrate the cuttings were mildly wilted; some were transferred to tap water after $\frac{1}{4}$ inch of the base of the stem was cut off under water. After these cuttings had remained 24 hours in water, the size and appearance of the wilted, curled, and necrotic areas of the leaflets remained about the same, with no increase or decrease. Since there was no recovery, it is apparent that either the injured tissues were too far gone to recover or that the toxic agent was still present and active. The uninjured portions remained green and turgid. This suggested that wilting induced by filtrates from crown-gall bacteria was not due to a mechanical plugging of the vessels at the base of the stem. This idea was supported by the fact that only the leaflets were wilted by the filtrates from crown-gall bacteria, while the stems and petioles remained turgid. However, the possibility of a mechanical plugging in the leaflets remained.

Cuttings in egg albumin solution (0.4 per cent powder) were tested in a similar manner. In 24 hours both stems and leaves were severely wilted. About $\frac{1}{4}$ inch of the stem's base was cut off under water and some of the cuttings were placed in tap water. After another 24 hours these appeared normal, while those remaining in the albumin were extremely wilted. Probably the albumin plugged the vessels at the base of the stem.

### *The Nature of the Wilt-Inducing Substance*

In each of the tests described below filtrates or concentrates were treated in various ways. They were then assayed to determine any change in their wilt-inducing properties. Unless otherwise stated, all experiments were performed at least two times.

*Toxic substance and length of fermentation.*—Cultures of the virulent organism, A6, grown in the sucrose, urea, mineral-salts medium incubated for 0, 5, 10, and 15 days were filtered through a mat of asbestos and Hyflo Super-Cel and the filtrates assayed at 6 dilutions (Table 4). The amount of toxic substance in culture increased markedly during the first 5 days. However, the increments thereafter were not statistically significant. Thus, for later tests, cultures 5 to 8 days of age usually were employed.

*Fractionation in alcohol.*—Several fractions were obtained by treating the fermented media with alcohol by essentially the same procedure used for the separation of the polysaccharide studied earlier (18, 26). For example,

fermented media from two 8-day and one 10-day fermentations were pooled, making a total of 11.5 liters. The liquid was concentrated by distillation under reduced pressure to about 1/30 of the original volume and the concentrate was poured into 1 volume of absolute alcohol. A stringy precipitate of cells and "gum" (Ppt. I) settled at once and was removed by centrifugation. The clear supernatant was concentrated to approximately 200 ml. and was poured into 1.3 volumes of absolute alcohol. A precipitate (Ppt. II) formed and was collected by centrifuging. Ppt. I and Ppt. II were washed in 60 per cent alcohol, and the washings were added to the main supernatant liquid, which then was concentrated to about 200 ml. and was stirred into 2 liters of absolute alcohol. A white precipitate (Ppt. III, lot 1) separated and was allowed to settle for 2 hours or more before it was collected by centrifugation. The supernatant was concentrated to 125 ml. Ppt. III (lot 1) was dissolved in water and reprecipitated in 9 to 10 volumes of absolute alcohol.

TABLE 4.—*The relationship of toxic substance to length of fermentation*

| Dilution | Length of fermentation (days) | | | |
|---|---|---|---|---|
| | 0 | 5 | 10 | 15 |
| | *Toxic units per ml.*[a] | *Toxic units per ml.*[a] | *Toxic units per ml.*[a] | *Toxic units per ml.*[a] |
| 1.25 | 0.02 | 1.1 | 1.2 | 1.1 |
| 2.5 | 0.03 | 1.3 | 0.9 | 1.2 |
| 5.0 | 0.05 | 1.5 | 1.3 | 1.4 |
| 10 | 0 | 1.9 | 1.4 | 2.8 |
| 20 | 0 | 0.4 | 1.5 | 0.9 |
| 40 | 0 | 0.6 | 0.6 | 0.9 |
| Averages | 0.02 | 1.1 | 1.3 | 1.4 |

[a] Each figure is the average of 10 cuttings.

For further purification, Ppt. III (lot 1) was triturated in a mortar, first with 90 per cent alcohol, then with absolute alcohol. The washings from each trituration and the supernatant from the final precipitation were concentrated separately. The various precipitates were dried at 60° C. under reduced pressure over sulphuric acid. All fractions were then assayed for toxicity as recorded in table 5.

These data indicated that about 71 per cent of the toxic substance was precipitated in 90 per cent alcohol. However, in a similar fractionation of 2340 ml. of another lot of fermented medium, which was first supercentrifuged to remove bacterial cells and then autoclaved, the precipitate, Ppt. III (lot 2), which separated in 90 per cent alcohol, accounted for only 48 per cent, while the material soluble in 90 per cent alcohol accounted for 50 per cent of the toxic activity.

These rather large differences in the distribution of toxic substance may be related to differences in the amounts formed in the two runs or to variations in the fractionation procedure. In any event, it was clear that the fractions

precipitated by 90 per cent alcohol contained large quantities of the toxic material. These concentrates were stored as dry powders at room temperature for several months with no apparent loss of activity and were convenient for use in subsequent tests.

*Nonvolatility.*—A solution of the concentrate, Ppt. III (lot 2), was made strongly alkaline with sodium hydroxide and steam distilled; approximately 100 ml. of the distillate were collected. The residue was then acidified with sulphuric acid, again steam distilled, and approximately 100 ml. of distillate were collected. The distillation products showed no toxic activity. Neutral distillation products were not studied separately since they probably would be found with the alkaline distillates which contained no toxic activity. The toxic substance thus appeared nonvolatile.

Parallel results were secured with the attenuated culture.

TABLE 5.—*Distribution of toxic substance in various fractions of fermented crown-gall medium*

| Sample | Solubility in alcohol | Total activity per fraction | Weight of solids | Portion of | |
|---|---|---|---|---|---|
| | | | | Total solids | Total toxic activity |
| | | *Toxic units*[a] | *G.* | *Per cent* | *Per cent* |
| Fermented medium | | 28,580 | 115[b] | 100 | 100 |
| Ppt. I | Insoluble in 50 per cent | 1,435 | 15 | 13 | 5 |
| Ppt. II | Precipitated in 57 per cent | 1,402 | 6 | 5 | 5 |
| Ppt. III (lot 1) | Insoluble in 90 per cent | 20,250 | 72 | 72 | 71 |
| Solution IV | Soluble in 90 per cent | 4,510 | ..... | ..... | 16 |
| | Totals | 27,597 | 93 | 90 | 97 |

[a] Each value is the average of 4 cuttings.

[b] This value was estimated from yields of lyophilized filtrates which were approximately 1 g. per 100 ml.

*Dialyzability.*—Passage of the toxic agent through membranes was tested by placing 100 ml. of the fermented medium into a Visking cellophane tube closed at the lower end and dialyzing against running tap water. After 4 days the material left in the tube was entirely inactive. It is unlikely that the active substance was destroyed in tap water since it withstood autoclaving at pH 4 to 5.

In another experiment 100 ml. of fermented medium were dialyzed against 2.5 liters of distilled water. After 48 hours both the dialysate and residue were concentrated to 100 ml. and were assayed for toxic activity. Compared to the original fermented medium, which was simultaneously assayed, approximately 50 per cent of the toxic activity was found in the dialysate and 50 per cent in the residue. The toxic substance thus appeared

diffusible through membranes; this suggested that it had a relatively small molecular size.

*Solubility.*—The toxic substance was very water-soluble. Numerous attempts were made to extract the toxic substance from filtrates and concentrates with various organic solvents. Portions (0.2 g.) of Ppt. III (lot 2) were shaken with 10 ml. of each of the following: methyl alcohol, methyl alcohol made to 1 N acidity with concentrated hydrochloric acid, methyl alcohol-pyridine solution (9 : 1 by volume), methyl alcohol made to 1 N alkalinity with concentrated sodium hydroxide, methyl alcohol-water solution (9 : 1), methyl cellosolve, pyridine, dioxan, acetone, ethyl alcohol, butyl alcohol, benzene, chloroform, ethyl acetate, and petroleum ether. The mixtures were allowed to stand overnight and were filtered. The filtrates were evaporated to dryness on watch glasses. An appreciable amount of residue was left only by acidified methyl alcohol (19 parts methyl alcohol, 1 part concentrated hydrochloric acid), methyl cellosolve, butyl alcohol, and pyridine. Further tests were made with these solvents.

In such cases, 1 g. of the concentrate was triturated with the solvent in a mortar, the mixture was filtered, the filtrate was evaporated to dryness under reduced pressure, and the solid substances were taken up in distilled water. The extracted residue and the portion dissolved with solvent were both assayed for toxic activity. In all cases, the main activity was found in the fraction not soluble in the organic solvent. Similar results were obtained by extracting with ether, butyl alcohol, and benzene from solutions of Ppt. III (lot 2) in 0.1 N sodium hydroxide. Ether extracts from neutral or acidified solutions of Ppt. III (lot 2) or from fermented medium were also nontoxic. The solubility of the toxic substance in the common organic solvents thus appeared slight.

*Other tests.*—Attempts were made to concentrate and to purify the toxic substance through the use of adsorbents. While the toxic substance was partially adsorbed on Norit, suitable procedures for eluting the substance were not found, and this technique was abandoned.

Procedures with ion exchangers were also employed but were not found satisfactory, since recovery of the adsorbed toxic substance from the ion exchangers was low.

*Association of toxic substance and polysaccharide in fractions.*—The high toxicity of the preparation precipitated in 90 per cent alcohol and the failure to effect a separation of the toxic substance and the previously studied polysaccharide, which occurs in this fraction (18, 26), suggested an association between toxicity and polysaccharide content. Further evidence of such an association was sought.

The amount of toxic substance and polysaccharide in culture in relation to length of fermentation was studied. Analyses for polysaccharide were made on the cultures described in table 4. The analysis for polysaccharide was made as follows. Samples were analyzed for reducing sugar after hydrolysis in 0.5 N sulphuric acid at 100° C. for 10 minutes and for 2 hours.

Reducing values obtained from samples hydrolyzed 10 minutes were taken as unfermented sucrose and the difference in reducing sugar between the 10-minute and 2-hour hydrolyses was called polysaccharide. A 10-minute hydrolysis under these conditions hydrolyzed less than 1 per cent of the polysaccharide to reducing sugar.

The data (Table 6) indicated a rapid increase in polysaccharide during the first 5 days of fermentation and a much slower but definite increase dur-

TABLE 6.—*Sucrose utilization, polysaccharide and toxic substance production, and length of fermentation*

| Length of fermentation | Residual sucrose | Polysaccharide (as sucrose) | Toxic units[a] |
|---|---|---|---|
| *Days* | *Mg. per ml.* | *Mg. per ml.* | *Per ml.* |
| 0 | 19.90 | 0 | 0.02 |
| 5 | 1.14 | 3.11 | 1.1 |
| 10 | 0.32 | 3.63 | 1.3 |
| 15 | 0.25 | 3.91 | 1.4 |
| 20 | 0.21 | 4.18 | ..... |

[a] These values represent the average toxic units from table 4.

ing the next 15 days. The toxic substance likewise increased rapidly during the first 5 days but did not increase significantly through the next 10 days. Thus, there appeared to be a concomitant variation between polysaccharide and toxicity. Similar evidence was sought with dialysis experiments.

The distribution of toxic substance and polysaccharide upon dialysis of filtrate from virulent crown-gall bacteria was examined. A cellophane tube (about 1.1 inches in diameter and 0.00072 inches wall thickness) containing 100 ml. of the filtrate was suspended in a vessel containing 2.5 liters of distilled water and a few drops of chloroform as a preservative. After 48 hours both dialysate and residue were concentrated at room temperature under reduced pressure to 100 ml. Samples of untreated filtrate and the residue remaining in the tube were analyzed for polysaccharide and then were assayed for toxic activity. The results (Table 7) indicated that approxi-

TABLE 7.—*Distribution of toxic substance and polysaccharide after dialysis*[a]

| Sample | Polysaccharide (as sucrose) | Toxic substance[b] |
|---|---|---|
| | *Mg. per ml.* | *Toxic units per ml.* |
| Untreated filtrate | 5.9 | 0.8 |
| Residue | 2.8 | 0.4 |
| Dialysate | 2.8 | 0.4 |

[a] The residue and the dialysate were each concentrated and diluted to the volume of the untreated filtrate.

[b] Each value is the average of 10 cuttings.

mately one-half of both the toxic activity and the polysaccharide had diffused through the membrane. Again there appeared to be a quantitative correlation between toxic activity and polysaccharide.

*Toxicity of purified bacterial polysaccharide.*—Assays of previously purified polysaccharide preparations indicated that this substance had considerable toxic activity. A comparison of the activities of the crude concentrate and purified polysaccharide is given in table 8.

TABLE 8.—*Toxic activity of the crude concentrate and various purified glucosan preparations*

| Sample | Toxic units |
|---|---|
| | *Per gram* |
| Ppt. III (lot 1) (crude preparation) | 766[a] |
| Preparation No. 2[b] | 241[a] |
| Preparation No. 3 | 202[a] |
| Preparation No. 5 | 379[a] |
| Preparation No. 6 | 425[a] |
| Preparation No. 6 regenerated from the acetate | 374[c] |

[a] Each value is the average from assays at 2 dilutions, 0.4 and 0.8 g. per 100 ml., with 2 cuttings at each dilution.

[b] Purified glucosan preparations 2, 3, 5, and 6 were the same as those described by Hodgson, Riker, and Peterson (18) and have low ash and negligible nitrogen contents.

[c] Average value from 2 dilutions, 0.15 and 0.72 g. per 100 ml., with 4 cuttings at each dilution.

These data indicate greater activities for crude than for purified preparations. This was so for all tests in which crude and purified glucosan were assayed simultaneously. However, when all values from separate assays of Ppt. III (lot 1) were considered, several in the range of those for the purified preparations were found. Ppt. III (lot 1), assayed on 9 different occasions at concentrations of 0.4 and 0.8 per cent, had an average activity (28 cuttings) of 570 toxic units per gram; the range was 347 to 766. Ppt. III (lot 2) had an activity of 614 to 745 toxic units per gram in 4 assays at 0.8 to 4 per cent concentrations; the average was 669 (18 cuttings). The range for purified glucosan (4 preparations) was 202 to 425, and the average was 312 (16 cuttings). (The quantity of highly purified preparations was limited, hence extensive assays of these materials were not made.)

In order further to determine whether the toxic activity was due to the polysaccharide itself or to some impurity, a purified preparation of glucosan (from an attenuated strain, A6–6, of the crown-gall organism) was acetylated with acetic anhydride and pyridine. The acetylated product, which is insoluble in water and soluble in chloroform, was purified and its optical rotation and acetyl value determined. The specific rotation $[\alpha]_D^{20}$ was +59 to +63 ($c = 1$, chloroform), and the acetyl value was 43 per cent. The values for a purified preparation from a virulent strain, A6, of the crown-gall organism as given by McIntire, Peterson, and Riker (26) were $[\alpha]_D^{20} = +56$ to +58.5, and the acetyl value was 42 to 44 per cent, respectively. The acetylated glucosan was then hydrolyzed with 1 N potassium hydroxide in methyl alcohol, the regenerated polysaccharide was precipitated in 10 volumes of absolute alcohol, collected, dissolved in water, and reprecipitated. The precipitate was dried under reduced pressure over sulphuric acid at

60° C. and assayed for toxic activity in aqueous solution. Another sample of polysaccharide acetate was hydrolyzed as above. The hydrolysate was passed once through a column of the cation exchanger, Zeo-Karb-H,[3] to remove potassium and then, after the addition of a few drops of dilute sulphuric acid, was concentrated under reduced pressure to remove methyl alcohol and acetic acid. The final solution was diluted with water, adjusted to pH 3 with barium hydroxide, filtered, and assayed for toxic activity. The values obtained for each preparation were, respectively, 383 and 366 toxic units per gram, or an average of 374. A comparison with the original polysaccharide (Table 8) indicated that a slight, but probably not significant, loss in toxic activity had occurred.

Since the polysaccharide maintained its toxic activity through acetylation and regeneration from the acetate and since other tests had failed to separate the toxic activity from the polysaccharide fractions, it appeared that a large portion, at least, of the toxic activity of media fermented by the crown-gall organism was due to the polysaccharide molecule itself.

Additional evidence that the glucosan and the toxic substance are identical is deduced from their similarity of behavior to heating with 1 N hydrochloric acid at 100° C.; in two hours the activity of each is completely destroyed. This treatment completely hydrolyzes the polysaccharide to glucose, which is inactive in the plant test.

## DISCUSSION

The foregoing evidence indicates that upon fractionation in alcohol the toxic substance in the culture appears mainly in two fractions; one is soluble in 90 per cent alcohol and the other is insoluble. The fraction precipitated in 90 per cent alcohol is composed largely of glucosan; it is the main toxic component of this fraction. The composition of the fraction soluble in 90 per cent alcohol was not studied critically. However, it might contain small amounts of polysaccharide which were incompletely precipitated in 90 per cent alcohol. In addition, some lower molecular weight glucosans may be present in this fraction.

The variation encountered in plant materials contributed one of the main difficulties when assaying the various fractions for toxic potency. It was deemed necessary to standardize the methods of growing the plants and the temperature and the relative humidity of the test room. The values chosen (25° C. $\pm$ 2° and 70 per cent $\pm$ 10 per cent relative humidity) were such that cuttings in unfermented medium showed little or no wilting in the 48-hour test period. Even under standardized conditions the variation in toxicity of a given sample tested on different occasions was undesirably large. The toxic values of samples assayed simultaneously were more uniform. Because of previous treatment, some plants appeared to be less sensitive than others to the toxic substance.

A comparison of the toxicity of our preparation with the peptide ob-

[3] The authors wish to thank the Permutit Company for supplying this material.

tained by the Swiss workers (8, 28, 29) would be of interest, but an adequate comparison is difficult because the two preparations were tested by widely differing techniques and probably under very different conditions of temperature and relative humidity. The Swiss workers used excised leaflets and measured the time to effect a certain degree of wilting as an index of potency, whereas, we employed cuttings bearing 2 to 3 well-developed leaves and recorded the degree of wilting produced in 48 hours. In 30 to 60 hours 2 mg. of their substance plus 0.04 mg. of iron as ferric chloride in 7 ml. of water caused complete wilting of tomato leaflets. Without addition of the ferric chloride 100 to 150 hours were necessary. In our test, an intake of 5 ml. of solution containing 20 mg. of glucosan wilted cuttings to about 70 per cent of the maximum. Thus on the weight basis their preparation seems more toxic. However, when excised leaflets were substituted in our test for cuttings bearing 2 to 3 well-developed leaves, the former appeared to be much more sensitive than the latter. Therefore, any valid comparison of the two compounds cannot be drawn from present information.

The relationship, if any, of the toxic substance to gall formation deserves discussion. First, the question arises whether the toxic substance is produced by the organism when growing in plant tissue since wilting ordinarily is not observed in the crown-gall disease. The lack of wilting may be due to unfavorable conditions in the plant for polysaccharide production and subsequent distribution, *e.g.*, (a) the relatively small numbers of bacteria inside the gall (30), (b) the low concentration of sugar substrate in the plant, and (c) particularly the growth of these bacteria in tissues other than the water-conducting vessels (30) where the so-called wilt organisms grow best. Secondly, filtrates from an attenuated as well as a pathogenic strain of the crown-gall organism induce wilting. (Similar findings for other nonparasitic microorganisms have been reported, *e.g.*, 19, 27.) In the case of the crown-gall organism, the attenuated strain produces the same glucosan as the virulent strain (18). Thus filtrates from the attenuated culture would be expected to induce wilting. On this basis no relationship is at present apparent between the toxic substance and gall formation. However, this toxic substance cannot wisely be ruled out by present information. It is recognized that pathogenicity may depend not on a single character, but on a number of factors which are in suitable balance (31). The final conclusion on this question must await further investigation.

There is no intention to infer that the ability to induce a wilting of plant cuttings is a unique property of the polysaccharide from crown-gall bacteria. Experiments with various other polysaccharides that also induce wilting will be described in a subsequent paper.

## SUMMARY

Filtrates from crown-gall bacteria were found to induce in the leaflets of tomato cuttings and other plants a wilting followed by a necrosis. The active (or toxic) substance inducing the wilting was investigated.

Under standardized conditions the approximate quantity of toxic substance in filtrates was measured by an assay involving filtrate concentration, solution intake, and severity of wilting (toxic index). Various factors affected the assay, particularly temperature, humidity, condition of plants, and length of assay period.

The toxic substance was thermostable in neutral solution, labile when heated in strong acid solution, and was nonvolatile, soluble in water, relatively insoluble in most organic solvents and dialyzable.

After alcoholic fractionation of the filtrate, much of the toxic activity appeared in a fraction precipitated in 90 per cent alcohol and consisted largely of a previously studied glucosan.

Several tests indicated a direct concomitant variation between the toxic substance and the polysaccharide. Therefore, several pure preparations of glucosan from crown-gall bacteria were tested and found to induce wilting similar to that of the whole filtrate.

The evidence available points to the glucosan primarily as the toxic substance.

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# GROWTH AND VARIATION OF SIX PHYSIOLOGIC RACES OF ACTINOMYCES SCABIES ON DIFFERENT CULTURE MEDIA[1]

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The potato scab organism, *Actinomyces scabies* (Thax.) Güssow, is subject to innumerable chemical and physical influences that may affect the stability of its biotypes and its ability to infect and parasitize its host. Isolated factors that might influence the growth and variation of *A. scabies* on agar media, in soil, and on potatoes were studied. These factors were different carbon sources, fertilizer ratios, fungicides, soil fungus extracts, and soil types.

Six isolates of monosporous lines from mass cultures of original isolations were used. Isolates 11 and 39 originated from type-4 scab pustules[2] on Irish Cobbler potatoes, isolates 46 and 47 from type-1 and type-2 pustules, respectively, on tubers of selection 161.37–29.[3] Isolate 48 came from a type-3 pustule on a tuber of selection 42.32–1–2, while isolate 49 was isolated from a type-2 pustule on a tuber of selection 31.37.[4] These lines differed so decidedly in cultural characters on potato-dextrose agar that they were considered distinct races (Table 1).

TABLE 1.—*Description of six physiologic races of Actinomyces scabies when grown on potato-dextrose agar*

| Race | Pigmentation[a] of medium | Color[a] of aerial mycelium | Aerial mycelium | Topography |
|---|---|---|---|---|
| 11 | Lemon chrome | White | Abundant | Smooth |
| 39 | None | Warm buff | do | do |
| 46 | Anthracene purple | Light flesh | do | do |
| 47 | Indigo blue | Indigo blue | do | do[b] |
| 48 | Dusky brown | White | Sparse | do |
| 49 | Light orange yellow | Slate gray | Abundant | do |

[a] Pigmentation based on Ridgway (12).

[b] Aerial mycelium in uniform ring-like zones.

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[2] Classification of pustules based on that of Leach *et al.* (7).

[3] The numbered potato selections were furnished by Dr. F. A. Krantz of the Division of Horticulture, University of Minnesota.

[4] Isolate 39 originated in sandy soil at Grand Rapids, Minn. The other isolates were obtained from potatoes grown in peat at Coon Creek, near Anoka, Minn.

PATHOGENICITY TESTS

The pathogenicity of the 6 races on 10 potato varieties and selections was determined in the greenhouse. Six-inch pots of sterile peat soil (pH 6.9) were inoculated[5] with individual races one week prior to planting. Six seed tubers of each variety were soaked for 1 hour in 1:1000 mercuric chloride and then washed thoroughly before cutting and planting. Controls for each selection were in sterile, noninoculated peat soil. The temperature of the soil averaged approximately 25° C. during the experiment.

TABLE 2.—*Results of inoculating 10 potato selections with 6 isolates of Actinomyces scabies*

| Selection | Infection rating of races[a] | | | | | | Field reaction[b] |
|---|---|---|---|---|---|---|---|
| | 11 | 39 | 46 | 47 | 48 | 49 | |
| 26.37–30s | 5/2 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 2.0 |
| 6–1 | 0/0 | 0/0 | 0/0 | 0/0 | 5/2 | 0/0 | 1.0 |
| Arnica | 0/0 | 45/1 | 45/1 | 0/0 | 0/0 | 0/0 | 0.7 |
| Earlaine | 20/3 | t/1 | 80/1 | 0/0 | 0/0 | 0/0 | 4.0 |
| US41956 | 45/4 | 4/54 | 20/2 | 5/3 | 0/0 | 45/2 | 4.0 |
| 627–240 | 0/0 | 5/1 | 20/1 | 5/2 | 0/0 | t/1 | 2.8 |
| 528–194 | 0/0 | 0/0 | 0/0 | 45/1 | 0/0 | t/1 | 2.1 |
| Hindenburg | t/1 | 0/0 | 20/1 | 0/0 | 0/0 | 0/0 | 1.6 |
| 19.37–25s | t/1 | t/1 | 0/0 | 5/1 | 20/2 | t/1 | 3.0 |
| 26.37–26s | 80/3 | 20/3 | 45/3 | 0/0 | 80/3 | 45/3 | 3.0 |

[a] Prevalence/Severity. Prevalence is given in terms of the percentage of tuber area infected. Severity is given in terms of the mean scab rating. t = trace of infection.

[b] Mean scab type derived by obtaining the mean for the scab readings on individual tubers in each clone from 5-hill units.

Parasitic races of *Actinomyces scabies* have been demonstrated previously by DeBruyn (4), Leach *et al.* (8), and Schaal (13). The reactions of the 10 varieties or selections of potatoes to the 6 races used in the present study differed. Isolates 11, 39, and 46 were the most pathogenic in the greenhouse tests; isolate 48 was severe on selections 6–1, 19.37–25s, and 26.37–26s, and it was the only isolate to infect selection 6–1 (Table 2). Isolates 47 and 49 generally were less virulent than other isolates. From the data it appears that a definite specificity for resistance and virulence exists between host and pathogen.

GROWTH OF RACES ON VARIOUS CULTURE MEDIA

The growth of each race was studied on media containing 11 carbohydrates; 26 combinations of nitrogen, phosphorus, and potassium; 6 fungicides; extracts of 6 soil fungi; and 3 types of soil. The specific substances were added to a suitable basic medium of 1.2 per cent agar or to the modified basic medium of Jensen (6), and the media were adjusted to pH 7.0. The cultures were grown at room temperature. All readings were based on triplicate series of cultures in 250-ml. Erlenmeyer flasks.

*Growth on carbohydrate media.* Of the carbohydrate media, sucrose,

[5] Inoculum was prepared by washing with 200 ml. sterile water the scab cultures grown on agar slant in 250-ml. bottles.

cellulose, inulin, and maltose were the more favorable for the growth of most races (Table 3). With the exception of race 48, the results on sucrose supported Vaughn's observations (14) that different isolates of *Actinomyces scabies* did not differ in growth on a sucrose medium. According to Afanasiev (1), all parasitic cultures of *Actinomyces* can utilize sucrose.

Vaughn's results (14), showing that physiologic races of *A. scabies* generally grew well on a cellulose medium, are supported by the results of this test. Although no destruction of the cellulose was observed, it is possible that the experiment did not continue long enough to permit this action to become noticeable.

Although Jensen (6) did not find inulin of value for other species of *Actinomyces*, growth of the races in our tests was excellent on this medium. The abundant growth of all but race 48 on maltose medium agrees with observations by Jensen (6) and Waksman (15). Generally the more pathogenic isolates attained the greatest growth on glycerine media; however, race 47, though not exceptionally virulent in greenhouse tests, grew well on glycerine media.

*Growth on media containing different NPK ratios.* Aerial growth generally was retarded by an increase of nitrogen, phosphorus, and potash salts in the medium (Table 3). Nitrogen alone, or in medium quantities in the presence of small amounts of the other salts, favored the production of aerial mycelium by all races. On the other hand, potash, in excess of the other salts, retarded growth. Phosphorus alone, in excessive amounts, was generally favorable for growth, although some differential reaction between races was observed. Race 49 grew well on nearly all media, apparently because of a comparatively greater tolerance for high salt concentrations.

The differential growth of the races on the various media may indicate the possibility of changing the relative prevalence of individual races in the soil by the use of different fertilizers, dependent on the physiologic races present in the soil. However, no generalization can be made for all races.

*Growth on fungicide media.* Spergon and Thiosan had some degree of specificity in the inhibition of the growth of the different races (Table 3). Spergon inhibited the more pathogenic races 11 and 39, whereas Thiosan inhibited race 39. The other races had a differential reaction to the fungicides. The ability of race 47 to grow on a 1:10,000 $HgCl_2$ medium was a striking example of specificity, as there was no indication of growth on this medium by the other races. This supports Frutchey and Muncie (5), who found several strains of *Actinomyces scabies* that grew in saturated solutions of mercuric chloride in a tyrosinate liquid medium. However, Lieske (9) reported that aerobic forms of the organism failed to grow on a medium containing a 1:100,000 solution of $HgCl_2$. It is possible that the use of such compounds either as a soil or seed treatment may retard one race more than another.

No growth was observed on media containing 1:1000 solutions of Corona PD–7, Semesan Bel, or yellow mercuric oxide. This substantiates reports by other workers (2, 10).

TABLE 3.—*Growth of 6 physiologic races of Actinomyces scabies on different culture media*

| Medium and concentration (g./l.) | | Aerial growth ratings of races[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 11 | 39 | 46 | 47 | 48 | 49 |
| *Carbohydrates*[b] | | | | | | | |
| Levulose | 25.5 | 4 | 5 | 5 | 5 | 5 | 3 |
| Galactose | 25.5 | 5 | 5 | 5 | 5 | 5 | 4 |
| Mannose | 25.5 | 1 | 1 | 2 | 1 | 3 | 1 |
| Soluble starch | 26.1 | 2 | 1 | 3 | 1 | 4 | 1 |
| Lactose | 22.7 | 2 | 1 | 2 | 4 | 3 | 1 |
| Sucrose | 22.9 | 1 | 1 | 1 | 1 | 4 | 1 |
| Dextrose | 25.5 | 1 | 1 | 2 | 2 | 4 | 1 |
| Cellulose[c] | 26.1 | 1 | 1 | 1 | 1 | 4 | 1 |
| Inulin | 20.0 | 1 | 1 | 1 | 1 | 1 | 2 |
| Maltose | 22.8 | 1 | 1 | 1 | 1 | 4 | 1 |
| Glycerine | 30.8 | 2 | 1 | 3 | 2 | 4 | 3 |
| *NPK salts*[d] | | | | | | | |
| ($NaNO_3$, 43 per cent $P_2O_5$, KCl) | 0–0–0 | 1 | 1 | 1 | 1 | 2 | 1 |
| | 1–0–0 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 0–1–0 | 1 | 1 | 1 | 1 | 3 | 1 |
| | 0–0–1 | 1 | 1 | 5 | 1 | 1 | 1 |
| | 1–1–0 | 1 | 1 | 1 | 1 | 3 | 1 |
| | 0–1–1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 1–0–1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 1–1–1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 10–1–1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 20–1–1 | 1 | 1 | 1 | 4 | 2 | 1 |
| | 1–10–1 | 1 | 1 | 3 | 1 | 1 | 1 |
| | 1–20–1 | 1 | 1 | 1 | 1 | 2 | 1 |
| | 1–1–10 | 4 | 1 | 1 | 2 | 2 | 1 |
| | 1–1–20 | 3 | 1 | 1 | 3 | 3 | 1 |
| | 10–10–1 | 1 | 3 | 2 | 1 | 4 | 1 |
| | 20–10–1 | 3 | 2 | 1 | 4 | 4 | 1 |
| | 10–20–1 | 3 | 3 | 4 | 4 | 4 | 1 |
| | 10–1–10 | 3 | 2 | 5 | 3 | 3 | 1 |
| | 10–1–20 | 4 | 3 | 2 | 3 | 3 | 3 |
| | 1–10–10 | 3 | 2 | 1 | 3 | 3 | 1 |
| | 1–10–20 | 4 | 3 | 1 | 2 | 4 | 1 |
| | 20–20–1 | 3 | 2 | 2 | 3 | 4 | 1 |
| | 20–1–20 | 4 | 2 | 1 | 4 | 4 | 1 |
| | 1–20–20 | 3 | 3 | 2 | 4 | 4 | 1 |
| | 20–20–20 | 4 | 2 | 2 | 3 | 4 | 4 |
| | 10–10–10 | 3 | 2 | 2 | 1 | 3 | 1 |
| *Fungicides*[e] | | | | | | | |
| Spergon | 1 : 1000 | 5 | 5 | 2 | 2 | 2 | 4 |
| Thiosan | do | 4 | 5 | 2 | 3 | 4 | 4 |
| Semesan Bel | do | 5 | 5 | 5 | 5 | 5 | 5 |
| Corona PD–7 | do | 5 | 5 | 5 | 5 | 5 | 5 |
| HgO (yellow) | do | 5 | 5 | 5 | 5 | 5 | 5 |
| $HgCl_2$ | 1 : 10,000 | 5 | 5 | 5 | 3 | 5 | 5 |
| *Soil fungus extracts*[f] | | | | | | | |
| *Melanconium putredinis* Wallr. | | 4 | 4 | 4 | 4 | 4 | 4 |
| *Aspergillus niger* T. & C. | | 4 | 4 | 4 | 4 | 2 | 3 |
| *Trichoderma lignorum* (Tode) Harz | | 5 | 4 | 4 | 4 | 4 | 5 |
| *Penicillium humicola* Oudem. | | 4 | 4 | 4 | 4 | 4 | 4 |
| *P. digitatum* Sacc. | | 4 | 4 | 4 | 4 | 5 | 4 |
| *Rhizoctonia solani* Kühn | | 3 | 4 | 4 | 4 | 4 | 4 |
| *Soil extracts*[g] | | | | | | | |
| Sand | | 3 | 3 | 3 | 4 | 4 | 3 |
| Peat | | 4 | 4 | 2 | 2 | 4 | 4 |
| Clay loam | | 4 | 4 | 4 | 4 | 4 | 4 |

*Growth on media containing extracts of soil fungi.* It is known (16) that competition with other micro-organisms limits the development of Actinomycetes in the soil. In this experiment only two of the fungus extracts tested had complete inhibitory effect on the growth of the Actinomycete (Table 3). *Penicillium digitatum* extract inhibited race 48, whereas extracts of *Trichoderma lignorum* inhibited races 11 and 49. The latter results support Daines' observations (3) that *T. lignorum* produces a diffusible substance that is toxic to *Actinomyces scabies.* The other extracts were detrimental to the growth of the isolates except for the effect of *Aspergillus niger* extract on race 48.

*Growth on media containing soil extracts.* Peat extract favored races 46 and 47 (Table 3), whereas races 11, 39, and 46 grew better on sand extract. It may be possible that the chemical nature of a soil may have a selective effect, allowing some races to grow better than others.

Pigmentation on all media indicated that this character, used by Millard and Burr (11), was a poor criterion for the classification of different species of *Actinomyces.* The extreme variability of this character observed in this investigation seemed to limit its use as a specific character.

## VARIATION

Variation among races of *Actinomyces scabies* is common (11, 13), but little is known concerning the factors that may influence the genetic stability of the organism. As nutritional factors might influence the stability of the organism, the frequency of variation and types of variants produced were studied on the media used for studying the characters of growth.

Before the experiments had progressed far it was apparent that certain races often produced variants culturally similar to other variants, both in the same and in other races. Whenever they arose, these variants were constant in their cultural characteristics when transferred to potato-dextrose agar and they were classified into types (Table 4).

*Variation on carbohydrate media.* Variation was influenced considerably by the carbohydrate media (Table 5). Mannose, starch, and sucrose caused considerable sectoring by all races, yet very little sectoring occurred on the cellulose medium. The low figures for levulose and galactose in the table were due to the absence of growth on these media.

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[a] Aerial growth: 1, excellent; 2, good; 3, fair; 4, poor; 5, none.

[b] Basic medium after Jensen (6), modified: potassium phosphate ($KH_2PO_4$), 2.0 g.; magnesium sulphate ($MgSO_4$), 0.5 g.; sodium chloride (NaCl), 0.5 g.; agar, 12 g.; distilled water, 1000 ml.

[c] Cellulose obtained by macerating filter paper and suspending the material in the basic medium.

[d] Basic medium 12 g. agar in 1000 ml. distilled water.

[e] Basic medium was Jensen's modified medium: salts and agar were added to 1 liter solution of fungicide. Media sterilized intermittently in flowing steam 1 hour for 3 days.

[f] Fungi were grown on potato broth which was then filtered under aseptic conditions through a sintered glass bacteriological filter into melted agar. Basic medium: 12 g. agar in 1 liter extract.

[g] Basic medium and preparation as for the soil fungus media.

TABLE 4.—*Frequency and cultural characters of variant types produced by 6 physiologic races of Actinomyces scabies*

| Variant type | Pigmentation[a] | | Aerial growth[c] | Topography[d] | Frequency[e] |
|---|---|---|---|---|---|
| | Medium[b] | Mycelium | | | |
| 1 | None | None | − | S | 89 |
| 2 | Apricot yellow | Apricot yellow | − | S | 10 |
| 3 | None | White | + | S | 65 |
| 4 | Salmon orange | Dark gull gray | + | S | 12 |
| 5 | None | Cream | + | R | 9 |
| 6 | do | Lavender gray | + | R | 4 |
| 7 | do | Warm buff | + | S | 15 |
| 8 | do | Ochraceous salmon | − | S | 3 |
| 9 | do | Dark gull gray | + | S | 2 |
| 10 | Dark grayish-olive | do | + | S | 1 |
| 11 | None | Deep gray olive | − | S | 12 |
| 12 | Deep cadet blue | Deep cadet blue | + | S | 3 |
| 13 | None | White | + | S | 396 |
| 14 | do | Neutral red | − | S | 2 |
| 15 | do | Orange chrome | − | S | 2 |
| 16 | Indigo blue | Indigo blue | − | S | 9 |
| 17 | Garnet brown | Violet gray | + | S | 13 |
| 18 | Deep cadet blue | Dark Corinth. purple | + | S | 1 |
| 19 | None | Parula blue | − | S | 1 |
| 20 | Ochraceous buff | Ochraceous buff | − | S | 9 |
| 21 | Dark Corinth. purple | Bordeaux | − | S | 5 |
| 22 | do | Deep cadet blue | + | S | 5 |
| 23 | do | Cream | + | S | 8 |
| 24 | Neutral red | White | + | S | 9 |
| 25 | Indigo blue | Xanthene orange | − | W | 1 |
| 26 | None | Indian lake | − | S | 1 |
| 27 | do | Scarlet | − | S | 1 |
| 28 | Indigo blue | Light cadet blue | + | S | 6 |
| 29 | Dull violet black | Pallid mouse | + | S | 17 |
| 30 | Indigo blue | Vinaceous drab | + | S | 7 |
| 31 | None | Ochraceous buff | − | S | 51 |
| 32 | Blanc's blue | Light cadet blue | + | S | 8 |
| 33 | None | Bluish black | − | S | 5 |
| 34 | do | White | − | S | 6 |
| 35 | do | Colorless | − | R | 1 |
| 36 | do | Cream | − | S | 5 |
| 37 | do | Mummy brown | − | R | 1 |
| 38 | do | Orange | − | S | 3 |
| 39 | do | Lemon yellow | − | R | 11 |
| 40 | Dull violet | Gull gray | ∓ | S | 114 |
| 41 | None | Lemon yellow | + | S | 5 |
| 42 | Dull violet black | White | + | S | 10 |
| 43 | Scarlet | Lemon yellow | − | R | 4 |
| 44 | do | Gull gray | + | S | 63 |
| 45 | do | White | + | S | 124 |
| 46 | None | Buff yellow | − | S | 20 |
| 47 | do | Bittersweet orange | − | S | 11 |
| 48 | do | Pinard yellow | − | R | 13 |
| 49 | do | Gull gray | + | S | 151 |
| 50 | Pyrite yellow | Pyrite yellow | − | S | 8 |
| 51 | Lemon yellow | Dark gull gray | + | S | 1 |
| 52 | Salmon orange | White | + | S | 11 |
| 54 | None | Lemon chrome | + | S | 4 |
| 55 | Ochraceous buff | Hermosa pink | + | S | 9 |
| 56 | Indigo blue | Indigo blue | + | S | 13 |
| 57 | Eugenia red | Hermosa pink | + | S | 43 |
| 58 | Lemon yellow | Black | + | S | 1 |
| 59 | Indigo blue | Gull gray | + | S | 1 |
| 60 | Chestnut brown | White | + | S | 1 |

TABLE 4.—(*Continued*)

| Variant type | Pigmentation[a] | | Aerial growth[c] | Topography[d] | Frequency[e] |
|---|---|---|---|---|---|
| | Medium[b] | Mycelium | | | |
| 61 | Lemon yellow | Gull gray | + | S | 4 |
| 62 | Cadmium yellow | Tawny | − | R | 1 |
| 63 | Light orange yellow | Slate gray | + | S | 1 |
| 64 | Dull violet black | Gull gray | + | R | 1 |
| 65 | None | Ochraceous tawny | − | W | 1 |
| 66 | do | Mouse gray | + | S | 12 |
| 67 | Garnet brown | Neutral gray | + | S | 53 |
| 68 | Honey yellow | Smoke gray | + | S | 1 |
| 69 | Neutral red | Deep olive buff | + | S | 59 |
| 70 | Lemon yellow | Pale bluegreen gray | + | S | 1 |
| 71 | Diamin-azo blue | Nigrosin blue | + | S | 1 |
| 72 | Lemon yellow | White | + | S | 3 |
| 73 | Blackish brown | Slate gray | + | S | 1 |
| 74 | Pale flesh | Pale flesh | + | S | 10 |
| 75 | Eugenia red | Gull gray | + | S | 25 |
| 76 | None | Sooty black | − | R | 3 |
| 77 | do | Ivory yellow | + | S | 12 |
| 78 | Dark Varley's gray | French gray | + | S | 3 |
| 79 | Carmine | Eosine pink | + | S | 6 |
| 80 | None | Slate gray | + | S | 30 |
| 81 | Neutral red | Flesh | + | S | 32 |
| 82 | Corinthian purple | Dark gull gray | + | S | 11 |
| 83 | Indian red | Light buff | + | S | 135 |
| 84 | Light orange yellow | Pale pink buff | + | S | 16 |
| 85 | Deep slate olive | White | + | S | 3 |
| 86 | None | Smoke gray | + | S | 6 |
| 87 | Cadmium yellow | Cream buff | + | S | 34 |
| 88 | Bishop's purple | do | + | S | 27 |
| 89 | None | Dark olive gray | + | S | 6 |

[a] Pigmentation is based on Ridgway's "Color Standards and Color Nomenclature" (12).
[b] Pigment diffused into the medium from the colony.
[c] Indicates presence (+) or absence (−) of aerial growth.
[d] S, smooth; R, rough; W, wrinkled.
[e] The total number of times each variant was observed in 918 chances.

*Variation on NPK media.* The effect of different NPK ratios on the frequency of sectoring was variable (Table 5), and no conclusions could be drawn. However, certain races were less stable on media in which the salts were present in small quantities. In the field this might lead to the formation of new races in the presence of fertilizers used at low concentrations.

*Variation on fungicide media.* The fungicide media had little effect upon the stability of the organism. Only one sector appeared in race 46 on Thiosan medium (Table 5); no sectors appeared on the other media. Consequently, the use of these compounds may have little influence on the formation of new races in the field.

*Variation on media with soil-fungus extracts.* Extracts of 6 soil fungi influenced the stability of races 48 and 49. Race 48 produced 2 sectors of variant type 8 on *Aspergillus niger* extract medium (Table 5), and race 49 produced a variant each on *Penicillium digitatum* and *Rhizoctonia solani* extract media. Thus, on extracts of these soil fungi new races arose. Al-

TABLE 5.—*Effect of media on the rate of variation among 6 physiologic races of Actinomyces scabies*

| Medium | Variation among races[a] | | | | | | Total |
|---|---|---|---|---|---|---|---|
| | 11 | 39 | 46 | 47 | 48 | 49 | |
| *Carbohydrates* | | | | | | | |
| Levulose | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 8/4 | 8/4 |
| Galactose | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Mannose | 0/0 | 12/1 | 18/4 | 2/1 | 4/4 | 127/2 | 156/12 |
| Soluble starch | 1/1 | 7/2 | 14/6 | 19/3 | 9/2 | 30/4 | 60/18 |
| Lactose | 24/2 | 27/1 | 9/3 | 0/0 | 10/1 | 88/2 | 48/9 |
| Sucrose | 1/1 | 37/2 | 10/4 | 10/3 | 2/1 | 147/3 | 207/14 |
| Dextrose | 5/3 | 0/0 | 0/0 | 6/2 | 5/3 | 4/1 | 18/11 |
| Cellulose | 0/0 | 0/0 | 5/1 | 3/1 | 0/0 | 8/1 | 16/3 |
| Inulin | 0/0 | 5/1 | 6/1 | 1/1 | 5/1 | 5/2 | 22/6 |
| Maltose | 0/0 | 11/1 | 2/1 | 3/1 | 2/2 | 117/2 | 135/7 |
| Glycerine | 0/0 | 1/1 | 0/0 | 3/1 | 8/1 | 23/3 | 35/8 |
| *NPK ratios* | | | | | | | |
| 0–0–0 | 0/0 | 0/0 | 26/4 | 6/2 | 8/1 | 16/1 | 56/8 |
| 1–0–0 | 1/3 | 1/1 | 9/4 | 6/2 | 2/1 | 0/0 | 21/9 |
| 0–1–0 | 0/0 | 1/1 | 10/1 | 12/2 | 0/0 | 3/2 | 26/6 |
| 0–0–1 | 0/0 | 1/1 | 0/0 | 11/4 | 3/1 | 3/1 | 18/7 |
| 1–1–0 | 2/2 | 4/2 | 28/4 | 15/1 | 3/1 | 15/2 | 67/12 |
| 0–1–1 | 14/2 | 1/1 | 16/2 | 12/1 | 11/1 | 25/2 | 79/9 |
| 1–0–1 | 22/1 | 0/0 | 35/3 | 0/0 | 5/1 | 3/1 | 65/6 |
| 1–1–1 | 9/1 | 11/1 | 11/2 | 19/2 | 5/1 | 0/0 | 55/7 |
| 10–1–1 | 0/0 | 0/0 | 9/1 | 11/1 | 2/1 | 0/0 | 22/3 |
| 20–1–1 | 0/0 | 12/1 | 0/0 | 0/0 | 3/1 | 2/1 | 17/3 |
| 1–10–1 | 0/0 | 0/0 | 0/0 | 0/0 | 12/1 | 0/0 | 12/1 |
| 1–20–1 | 0/0 | 0/0 | 0/0 | 9/2 | 0/0 | 0/0 | 9/2 |
| 1–1–10 | 22/1 | 4/1 | 3/1 | 22/3 | 3/1 | 5/1 | 59/8 |
| 1–1–20 | 0/0 | 9/1 | 0/0 | 12/1 | 0/0 | 0/0 | 21/2 |
| 10–10–1 | 9/2 | 3/1 | 0/0 | 0/0 | 0/0 | 60/1 | 72/4 |
| 20–10–1 | 0/0 | 0/0 | 3/1 | 0/0 | 0/0 | 0/0 | 3/1 |
| 10–20–1 | 11/2 | 0/0 | 0/0 | 9/1 | 0/0 | 0/0 | 20/3 |
| 10–1–10 | 8/1 | 8/1 | 0/0 | 6/1 | 0/0 | 0/0 | 22/3 |
| 10–1–20 | 8/1 | 3/1 | 0/0 | 3/1 | 0/0 | 0/0 | 14/3 |
| 1–10–10 | 11/1 | 0/0 | 4/2 | 4/1 | 3/1 | 0/0 | 22/5 |
| 1–10–20 | 7/1 | 0/0 | 0/0 | 9/1 | 0/0 | 0/0 | 16/2 |
| 20–20–1 | 0/0 | 11/1 | 0/0 | 9/1 | 0/0 | 17/2 | 37/4 |
| 20–1–20 | 0/0 | 0/0 | 5/1 | 2/1 | 0/0 | 33/1 | 40/3 |
| 1–20–20 | 11/1 | 11/1 | 9/1 | 11/3 | 0/0 | 0/0 | 42/6 |
| 20–20–20 | 7/1 | 0/0 | 0/0 | 2/1 | 0/0 | 3/1 | 12/3 |
| 10–10–10 | 9/1 | 11/1 | 3/1 | 5/1 | 0/0 | 8/1 | 36/5 |
| *Fungicides*[b] | | | | | | | |
| Thiosan | 0/0 | 0/0 | 1/1 | 0/0 | 0/0 | 0/0 | 1/1 |
| *Soil fungus extracts*[c] | | | | | | | |
| *Aspergillus niger* | 0/0 | 0/0 | 0/0 | 0/0 | 2/1 | 0/0 | 2/1 |
| *Penicillium digitatum* | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 4/1 | 4/1 |
| *Rhizoctonia solani* | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 2/1 | 2/1 |
| *Soil extracts* | | | | | | | |
| Sand | 9/1 | 0/0 | 0/0 | 0/0 | 0/0 | 1/1 | 10/1 |
| Peat | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 16/1 | 16/1 |
| Loam | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 24/1 | 24/1 |
| Totals | 111/29 | 181/25 | 236/49 | 232/46 | 110/30 | 777/46 | 1647/238 |

[a] Total number of sectors/total number of variant types.

[b] No sectors were observed on Spergon, Semesan Bel, Corona PD–7, $HgCl_2$, or HgO media.

[c] No sectors were observed on media containing extracts of *Melanconium putredinis*, *Trichoderma lignorum*, or *Penicillium humicola*.

though these cultural studies are not a definite indication of field reaction, the effect of certain soil fungi on *Actinomyces scabies* in culture emphasizes the importance of considering such a phenomenon in the field.

*Variation on soil-extract media and in soils.* The rate of mutation of most races was not influenced by extracts from different soils (Table 5); but race 49 was variable on extracts of peat and of clay loam, and race 11, the most stable of all the races studied, produced 9 sectors on sand extract. These results indicated that the chemical nature of the soil may influence the genetic stability of certain races, and such an influence must be considered as a factor in the formation of new races in the field.

Experiments with the different races in soils containing a mixture of the six soil fungi showed that the soil organisms had little effect on the less pathogenic races. The most striking results (Table 6) showed that during one year all races were more stable on peat soil than in sand, on the basis of

TABLE 6.—*The effect of soil organisms on the stability of 6 physiologic races of Actinomyces scabies growing in 2 types of soil*

| Series | Amount of variation[a] | | | | Total no. colonies |
|---|---|---|---|---|---|
| | On sand after | | On peat after | | |
| | 2 mo. | 12 mo. | 2 mo. | 12 mo. | |
| 11 | 0/0 | 1/45 | 0/0 | 0/0 | 1 |
| 11 +[b] | 0/0 | 0/0 | 0/0 | 0/0 | 0 |
| 39 | 0/0 | 1/45 | 0/0 | 0/0 | 1 |
| 39 + | 0/0 | 0/0 | 1/4 | 0/0 | 1 |
| 46 | 0/0 | 20/45 | 0/0 | 2/45 | 22 |
| 46 + | 0/0 | 0/0 | 0/0 | 2/45 | 2 |
| 47 | 0/0 | 26/45 | 0/0 | 3/45 | 29 |
| 47 + | 1/5 | 10/45 | 0/0 | 0/0 | 11 |
| 48 | 0/0 | 3/66 | 0/0 | 1/45 | 4 |
| 48 + | 0/0 | 0/0 | 0/0 | 0/0 | 0 |
| 49 | 2/13 | 16/13 | 2/13 | 2/13 | 22 |
| 49 + | 0/0 | 0/0 | 1/13 | 0/0 | 1 |
| Total no. colonies | 3 | 77 | 4 | 10 | 94 |

[a] Number of colonies/variant type.
[b] + denotes inoculation of the soil with 6 soil organisms.

colonies present in the dilution plates. Except for variant type 13, the variant types isolated after 2 months usually were not obtained after 12 months. The variant types 4 and 5 were weak variants which failed to grow upon repeated transfers to potato-dextrose agar. The predominance of variant type 45 after 12 months was very striking and is difficult to explain. Stock cultures were carried in peat soil after this test had been made, because the physiologic races seemed to maintain their stability better in peat than on nutrient agar or in sand. This fact may offer an explanation for the variability of scab reaction among different types of soils. The relative stability of the races in the presence of the other soil organisms is not significant, as competition by these soil organisms could have prevented the establishment of many variants in the soil.

*Variation on the host.* When the different races of *Actinomyces scabies* were reisolated from the tubers at the conclusion of the pathogenicity tests, several variants appeared in the dilution plates (Table 7). Variant type 4 was isolated from tubers inoculated with races 11, 47, and 49, respectively.

TABLE 7.—*Variation of 6 races of Actinomyces scabies arising from scab pustules in pathogenicity tests on 7 potato selections*

| Potato selections | Variation of races[a] | | | | | |
|---|---|---|---|---|---|---|
| | 11 | 39 | 46 | 47 | 48 | 49 |
| 6–1 | ..... | ..... | ..... | ..... | 2/31 | ..... |
| Earlaine | ..... | ..... | 1/17<br>1/22 | ..... | ..... | ..... |
| US 41956 | 4/4 | ..... | ..... | 3/4 | ..... | 2/39<br>2/52 |
| 627–240 | ..... | 1/10 | ..... | ..... | ..... | ..... |
| 528–194 | ..... | ..... | ..... | ..... | ..... | 1/39 |
| 19.37–25s | 1/4 | ..... | ..... | ..... | 2/31 | 1/4<br>1/39 |
| 26.37–26s | 3/4 | ..... | ..... | ..... | 3/39 | 3/39 |

[a] Pustule type/variant type.

In all, this variant was isolated 5 times from 3 host selections. It was not associated with any particular pustule type, being found in a type-1 pustule on selection 19.37–25s, in a type-3 pustule on selection 26.37–26s, and in pustules of type-3 and type-4 on selection US 41956. These pustule types were characteristic of the reaction of the parent race on each selection; since nothing is known of the pathogenicity of the variants, it was assumed that the symptoms were produced by the parent race. This is further substantiated by the fact that the parent races were also isolated from the same pustules, and in much greater abundance than were the variants. It was unlikely that these "variants" were contaminants, because the absence of scab on check plants showed that such contamination was rare. Apparently variation in the organism while living parasitically on the host is a rather common occurrence.

The results of the different tests described in this paper indicate that the more pathogenic races, 11 and 39, were also the more stable. Because of the poor growth of race 48, although comparatively few sectors were produced, it was considered less stable. The other races sectored rather frequently and often produced the same variant types (Table 5), which might indicate a close genetic relationship among races. Some variant types were predominant in a single race, such as type 83 in race 11 and type 49 in race 49.

## DISCUSSION

Growth and genetic stability of physiologic races of *Actinomyces scabies* on agar media are influenced by different balances of carbohydrates and salts. Such balances, either in the host or in the soil, may possibly influence

the presence of certain races, and the production of new races. The apparent differences in growth among races of various carbohydrates may indicate the ability of some races to survive better as saprophytes than others.

Although many workers have found that fertilizers affect the incidence of scab infection, this study indicates that the ratio between nitrogen, phosphorus, and potash is not consistent in its effect on all physiologic races.

In general, the fungicides tried had no pronounced effect on the genetic stability of the organism, but distinct differential growth reactions were observed on Spergon and Thiosan media. As these two compounds are generally used in seed treatment, because of their specificity toward certain organisms, their specific effects on certain physiologic races of *A. scabies* are consequently of some interest. The ability of race 47 to grow on mercuric chloride media indicated that the use of this compound as a seed disinfectant may not mean necessarily that the organism on the seed tuber will be killed. Under field conditions the occurrence of such a reaction is open to question; however, the possibility of seed treatment fungicides exerting a selective effect on physiologic races of *A. scabies* in the field should be investigated further before there can be assurance that a specific compound can control the disease.

Experiments with other soil fungi in relation with the physiologic races of *A. scabies* showed not only that certain organisms may inhibit or produce a differential effect on the growth of these races on agar media, but also that other organisms may upset the genetic stability of certain races. It seems probable that fluctuations in the incidence of scab in different areas may often be due to the presence of certain microflora which could either favor the growth of some races of the scab organism to the exclusion of others or influence the formation of new races through variation.

Results with soil-extract media and soil types indicated that some races can survive better in one soil than in another. Although the data presented herein are not conclusive, they open an interesting field of investigation. That soil types have a pronounced influence on the genetic stability of the organism is of extreme importance. In many sandy areas the scab reaction on a given potato variety is variable, the pustule types ranging from 1 to 4. On the other hand, in many peat soils scab reaction is more or less constant, with little variation in the pustule type. The effects of soil type may be dependent to some extent on the antagonistic action of the soil microflora on the scab organism.

The variation of the organism while living parasitically on the host is of considerable interest, as it offers another possibility for the introduction of new races of *A. scabies* into the soil. Thus, it is evident that the growth and genetic stability of these races are dependent on a complex interrelationship of physical, chemical, and biological factors. Any deviation of these factors may be capable of favoring the survival of some races to the exclusion of others, or the formation of new races.

SUMMARY

Six physiologic races of *Actinomyces scabies* (Thax.) Güssow were distinctly different in pathogenicity on ten different potato varieties or selections.

Sucrose, cellulose, inulin, and maltose media were the most favorable sources of carbon for the growth of these races.

Increasing amounts of nitrogen, phosphorus, and potash retarded the production of aerial mycelium by most races. Nitrogen and phosphorus were generally favorable for growth; potash tended to retard it.

Spergon and Thiosan, at the concentration of 1:1000, were specific in their effect on the growth of the different races; one race grew on media containing a 1:10,000 dilution of mercuric chloride. No growth was observed on media containing 1:1000 dilutions of Corona PD–7, Semesan Bel, or yellow mercuric oxide.

*Trichoderma lignorum* extract inhibited the growth of 2 races, and 1 race failed to grow on a medium containing an extract of *Penicillium digitatum*. Other soil organisms studied had no effect on growth.

Maximum growth and stability were observed on peat soil; mineral soils tended to retard or inhibit growth and increase variability in the races studied.

The more pathogenic races were most stable on most media.

Variants were produced by some physiologic races while living parasitically on the host.

Some variant types were peculiar to individual races, but certain types were produced frequently by several races, which seemed to indicate a close genetic relationship between those races.

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# RESISTANCE TO MEALYBUG WILT OF PINEAPPLE WITH SPECIAL REFERENCE TO A CAYENNE–QUEEN HYBRID[1]

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## INTRODUCTION

The control of mealybug wilt can be considered the major biological problem with which pineapple growers in Hawaii are concerned. Direct control measures against the insect [*Pseudococcus brevipes* (Ckl.)] have achieved a practical degree of success but only at great and continued expense. The development of a resistant variety, even if only moderately resistant, would be of value, either in reducing the cost of current control measures, or in improving the degree of control obtained at present. Testing of pineapple varieties indicated very early that there were varietal differences in susceptibility to mealybug wilt. Since then a number of varietal hybrid populations have been tested for susceptibility to wilt. From these tests it is now possible to report progress, first in establishing that resistance occurs, second in indicating something of the genetic aspect of the resistant character, and finally to reveal some of the unique aspects of the problem with which future studies must be concerned.

## PINEAPPLE VARIETIES USED IN HYBRIDIZATION

Eleven varieties of pineapples (Table 1) were studied with respect to susceptibility to mealybug wilt. These were collected from widely separated geographical areas and little is known of their origin or genetic relationships. Some of them can be placed into related groups on the basis of generally similar characters.

Cayenne is the variety grown commercially in Hawaii and a number of other countries, and is highly susceptible to mealybug wilt. Sarawak appears to be a strain of the Cayenne variety which had been grown in the province of Sarawak in northeast Borneo, and it is morphologically indistinguishable from Cayenne. Queen, a spiny-leaved, small-fruited variety grown in a number of countries, is perhaps one of the oldest named varieties of pineapple. Natal and MacGregor closely resemble Queen in their general appearance. Red Spanish is grown commercially in Florida and the West Indies where it has been considered more hardy and vigorous than Cayenne. Pernambuco is a hardy, spiny-leaved form grown in some parts of Brazil. Monte Lirio is a smooth-leaved, white-fleshed variety grown to some extent

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in Mexico and Central America, but it lacks growth vigor in Hawaii. Ruby is a smooth-leaved variety from British Malaya where it has been one of several cultivated forms. *Ananas ananassoides* is a hardy, small-fruited, thorny-leaved wild species distributed over a wide area in the dry regions of Brazil. Congo is a spiny-leaved, small-fruited variety from the Belgian Congo region of Africa and it has no characters of commercial value in Hawaii.

The resistant hybrid No. 7898 with which this report specifically deals was selected in 1928 from a Cayenne × Queen $F_1$ population. It is, in general, a mosaic of the characters of the two parents, with dominance of the Queen variety showing in fruit color and texture and in type of slips and suckers, but in fruit size it is more like Cayenne. The leaves of this hybrid, No. 7898, normally have less anthocyanin pigment than does the Cayenne parent. The chlorophyll color of the leaves is a light pea green in contrast to the darker sage green of the Cayenne variety. The hybrid also ripens its fruit from two to three weeks earlier than its Cayenne parent. Cayenne normally produces 1 to 2 suckers which produce the ratoon crop while the hybrid produces from 3 to 6 suckers.

## METHODS OF TESTING FOR RESISTANCE

The several methods used consisted essentially of exposing the plants to mealybug infestation. The first was designed to provide as equal an infestation as possible for each plant tested. In this method, mealybugs were collected from wilting areas in pineapple fields and the medium-sized specimens separated out.[3] These were accumulated *en masse*, and then lots of 50 were enclosed in vials and carried out to the field plots the following day, the insects being without food in the interim.

A second method was to use the rinds of heavily infested green fruits. These were collected one day and carried to the field the next, the pieces of rind bearing mealybugs being cut off just prior to depositing them on the plants to be infested. Since this method has been used also when great numbers of plants were to be infested, the fruits were cut and the pieces removed to the field plots in buckets. One piece was applied to the center of each plant. This method is useful where large numbers of plants are to be grossly infested, but the number of mealybugs actually applied by this method is often very large, and since the bugs must move from the fruit tissue as it dries up, there often is also movement away from the infested plant onto nearby checks.

A third method, much more severe than either of the others, consists of

[3] The term "wilting area" means a section of a pineapple field, usually on a border, where wilted plants are common, or predominant. The largest mealybug colonies are found in such circumstances on the apparently healthy plants, and it is from these that the mealybug-infested fruits are usually taken. If fruits on wilted plants are selected, colonies are usually smaller, and experience has shown that these colonies are no more efficient in inducing wilt than are those from nearby healthy plants; on the contrary some large-scale infestations made from mealybugs from these wilted plants have induced a disappointingly small incidence of wilt when transferred to test plants.

growing the plants in an area where natural infestation can occur and be left untouched by artificial control methods. Biological factors cannot of course be controlled, but in the presence of the ant, *Pheidole megacephala* Fab., these factors are seldom sufficient to prevent the maintenance of a mealybug colony.

A fourth method, the one most recently used, is to permit the establishment and maintenance of mealybugs on the variety and to make adjacent new plantings so that young plants could be infested naturally from the older infested planting of the same variety. Here the new plantings are infested with bugs which had been grown on the same variety. In the third method, the bugs which infest the new planting may have been developed upon a different variety.

### THE SUSCEPTIBILITY OF CAYENNE WHEN COMPARED WITH OTHER VARIETIES AND HYBRIDS

This was originally determined by the first method of testing, namely, to infest each plant with 50 mealybugs and to permit the infestation to operate only for about two weeks. It is clear from the results in table 1, that Cayenne is the most susceptible variety to be included in these tests. This finding is in accord with experience in other parts of the world where other varieties have replaced Cayenne as the commercial varieties, because of the susceptibility of Cayenne to mealybug wilt (2).

The susceptibility of variety hybrids was also determined by the first method of testing. From the results shown in table 2, it is evident that susceptibility is a dominant character in $F_1$ hybrids. As with Cayenne, there is considerable variation in the number of plants wilting following specific infestations (1, 4), and for that reason the data are arranged to show the percentage of hybrid plants wilting with an infestation which at the same time induced a given percentage of wilt in Cayenne.

### EXPERIMENTS WITH HYBRID NO. 7898 IN PLANTATION FIELDS

In these experiments No. 7898 was planted alongside Cayenne in plantation fields along borders where natural infestation was most likely to be

TABLE 1.—*Tests with pineapple varieties. Wilt following single infestations of 20 plants of each variety with 50 mealybugs per plant*

| Variety | Percentage of plants wilted when Cayenne wilted as follows: | | | | |
|---|---|---|---|---|---|
| | 16 | 30 | 40 | 50 | 60 |
| Red Spanish | 0 | 30 | ..... | 0 | ..... |
| *Ananas ananassoides* | 0 | ..... | ..... | ..... | ..... |
| Pernambuco | 10 | 0 | 0 | ..... | 15 |
| Queen | 2 | 0 | 0 | 0 | 10 |
| Sarawak | ..... | ..... | ..... | ..... | 0 |
| Congo | ..... | ..... | 5 | ..... | 5 |
| Natal | ..... | ..... | 0 | ..... | 0 |
| MacGregor | ..... | ..... | ..... | ..... | 20 |
| Ruby | ..... | ..... | ..... | ..... | 0 |
| Monte Lirio | ..... | ..... | ..... | 5 | ..... |

TABLE 2.—*Tests with Variety × Cayenne hybrids. Wilt following single infestations of 20 plants of each hybrid with 50 mealybugs per plant*

| $F_1$ hybrids from crossing Cayenne with following varieties | Percentage of hybrid plants wilted when Cayenne wilted as follows: | | | | | |
|---|---|---|---|---|---|---|
| | 10 | 20 | 30 | 50 | 60 | 70 |
| Red Spanish | ..... | ..... | 31 | ..... | ..... | ..... |
| *A. ananassoides* | 12 | 0 | ..... | 35 | ..... | 30 |
| *A. ananassoides* 3N | ..... | ..... | 30 | ..... | ..... | ..... |
| Ruby (Hybrid No. 8806) | ..... | ..... | 40 | 45 | ..... | ..... |
| Ruby[a] | ..... | ..... | ..... | 10 | ..... | ..... |
| Queen (Hybrid No. 7789) | ..... | ..... | ..... | ..... | 15 | ..... |
| do (Hybrid No. 7898) | ..... | ..... | 5 | ..... | ..... | ..... |
| Pernambuco (Hybrid No. 8594) | ..... | ..... | ..... | ..... | 20 | ..... |
| do (Hybrid No. 8597) | ..... | ..... | ..... | 25 | ..... | ..... |
| do (Hybrid No. 9166) | ..... | ..... | 20 | ..... | ..... | ..... |
| do (Hybrid No. 9243) | ..... | ..... | ..... | ..... | ..... | 25 |
| Monte Lirio (Hybrid No. 8728) | ..... | 20 | 40 | ..... | ..... | ..... |
| do (Hybrid No. 8752) | ..... | 10 | 10 | ..... | ..... | ..... |
| do (Hybrid No. 8817) | ..... | ..... | 0 | ..... | ..... | ..... |
| Natal (Hybrid No. 8971) | ..... | ..... | ..... | ..... | ..... | 25 |

[a] Sarawak used instead of Cayenne as one parent of hybrid.

encountered. In addition, plantings made in plantation fields for agronomic comparison furnished additional evidence when accidental infestation of mealybugs occurred. It was in these plantings that the most striking evidence for resistance under conditions of plantation practice was obtained. The visual differences were accentuated by the dense growth habit of No. 7898 when surplus suckers were not removed and critical observation revealed many of the No. 7898 plants having the typical mild symptoms often present in this hybrid.

### SYMPTOM EXPRESSION IN VARIETIES AND HYBRIDS

Factors influencing symptom expression are to be reckoned with in evaluating wilt incidence in hybrids and varieties other than Cayenne. Amount of anthocyanin materially affects the red and pink color reaction. In a broad-leaved succulent plant the downward drooping of the leaves characteristic of 3rd-stage wilt will be much more definite than in a stiff, narrow-leaved variety. In the narrow-leaved varieties, the inner reflexing of the leaf margins will be most pronounced; but so rigid and stiff is the leaf of some such varieties that symptoms rarely progress beyond the 2nd color stage, and downward bending of the leaves is limited to a dry distal portion. Varieties with pale green, almost-anthocyanin-free leaves, go through a sequence of yellow shades rather than red and pink as is the case with Cayenne. When differences in plant type and anthocyanin content are considered, however, there is normally no difficulty in diagnosis except in cases of extremely mild symptoms in varieties with which experience is limited.

Symptoms in hybrid No. 7898 are of special interest. This variety, as has been described, is low in anthocyanin and has a pea green color. It develops a lush wide-leaved growth as it approaches fruit maturity. The

suckers arising from the mother plant stem are numerous and tend to be narrow-leaved. Symptoms in this variety range from a slight yellowing of three or four adjacent median leaves to symptoms typical of Cayenne in every respect except for the development of the red and pink coloration and the progress to fourth stage in young plants, which occurs but rarely. There is a drying up of the fruit peduncle of this hybrid so that the fruit, as it matures to large size, cannot be supported and bends over (Fig. 1).

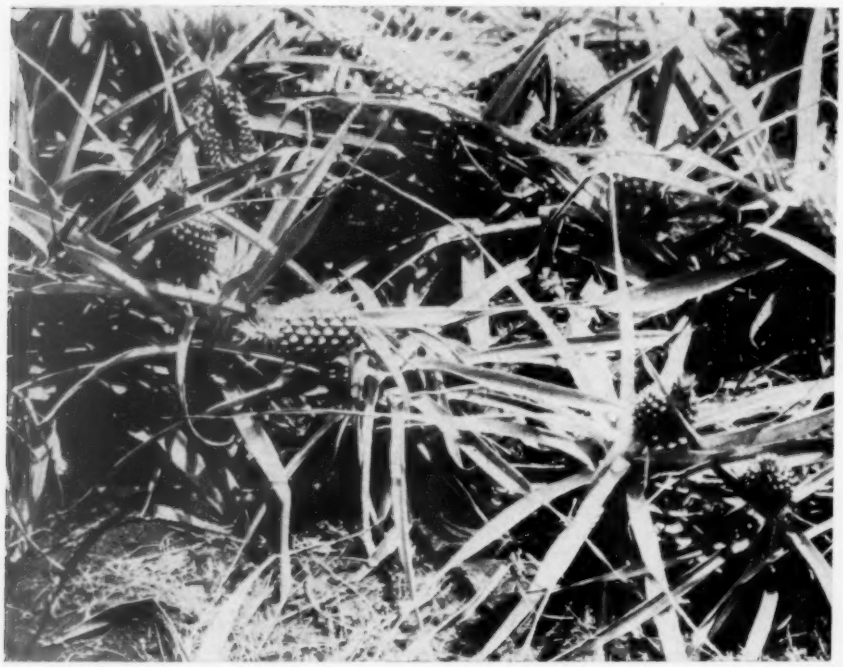

FIG. 1. Symptoms of wilt which occur in hybrid No. 7898 following several separate heavy infestations of mealybugs. Note the drooping fruit due to a drying peduncle.

## DETAILED EXPERIMENTS WITH HYBRID NO. 7898

With the accumulated evidence from several years experience of the resistance of No. 7898 to wilt, about one acre was planted to this variety in the fall of 1941 for tests along the following lines: The effect of monthly infestations beginning in February, 1942, and continuing until July, 1942; single infestations to determine the sub-wilting effect, if any, of mealybug feeding; the effect on fruit and first ratoon suckers of late infestations made at monthly intervals from August, 1942, to October, 1942; the effect of removing excess ratoons on susceptibility to ratoon infestation; uncontrolled natural infestations.

*Section 1. The effect of monthly infestations.* Infestations were applied as indicated in table 3, beginning in February, 1942, and continuing until July, 1942.

TABLE 3.—*The monthly infestations of hybrid No. 7898 and Cayenne in 1942*

| Variety and beds infested | Time of infestation |
|---|---|
| No. 7898 | |
| Beds 47–48 | Feb., Mar., Apr., May, June |
| Beds 49–50 | Mar., Apr., May, June |
| Beds 51–52 | Apr., May, June |
| Beds 45–46 | May, June |
| Beds 43–44 | June, July |
| Cayenne | |
| Bed 53 | Apr., May, June |

It was clear, however, from detailed mealybug counts taken in August, that the successively applied mealybug colonies had not become permanently established and that the results, therefore, were due to the cumulative effects of the separate infestations rather than to the accumulation of huge mealybug populations.

Symptoms on these plants were recorded at intervals, and in table 4 the data up to December 29 are presented. It will be noted that fairly large percentages of No. 7898 are recorded as showing symptoms but the symptom expression was of a decidedly mild type. Figure 2 shows No. 7898 and Cayenne, each infested at three successive intervals. As a matter of fact, severe wilt was already manifest in many Cayenne plants at the time the third infestation was made and the collapse in the Cayenne was as complete and

FIG. 2. On the right is Smooth Cayenne with severe wilt, on the left is hybrid No. 7898. Both beds had received three separate infestations of mealybugs.

severe as has ever been experienced. The photograph, while not adequate to indicate the symptom on No. 7898, is sufficiently clear to indicate an extreme difference in the susceptibility of the two varieties. The symptom in No. 7898 can best be described as a slight yellowing of a few, usually three or four, adjacent median leaves; that in Cayenne as typical 4th-stage (severe) wilt (4). Some No. 7898 plants progressed to 3rd-stage wilt but even this was usually limited to the original median leaves, the center of the plant growing out apparently normally while this progression was occurring.

TABLE 4.—*Wilt records from plants infested with mealybugs from 2 to 5 times at monthly intervals. Beds 43–52, No. 7898. Bed 53, Cayenne*

| Bed No. | Plot No. | No. of infestations | No. of plants in plots | Plants showing symptoms | |
|---|---|---|---|---|---|
| | | | | No. | Per cent |
| 43 | A | 2 | 100 | 33 | 33 |
| | B | 2 | 99 | 48 | 48 |
| | C | 2 | 100 | 63 | 63 |
| | D | 2 | 114 | 72 | 63 |
| 44 | A | 2 | 100 | 60 | 60 |
| | B | 2 | 100 | 44 | 44 |
| | C | 2 | 100 | 42 | 42 |
| | D | 2 | 106 | 54 | 51 |
| 45 | A | 2 | 100 | 40 | 40 |
| | B | 2 | 100 | 36 | 36 |
| | C | 2 | 100 | 42 | 42 |
| | D | 2 | 96 | 53 | 55 |
| 46 | A | 2 | 100 | 56 | 56 |
| | B | 2 | 100 | 50 | 50 |
| | C | 2 | 100 | 57 | 57 |
| | D | 2 | 83 | 43 | 52 |
| 47 | A | 5 | 100 | 69 | 69 |
| | B | 5 | 100 | 72 | 72 |
| | C | 5 | 100 | 75 | 75 |
| | D | 5 | 74 | 58 | 78 |
| 48 | A | 5 | 100 | 62 | 62 |
| | B | 5 | 100 | 68 | 68 |
| | C | 5 | 100 | 60 | 60 |
| | D | 5 | 65 | 41 | 63 |
| 49 | A | 4 | 100 | 70 | 70 |
| | B | 4 | 99 | 47 | 47 |
| | C | 4 | 99 | 69 | 70 |
| | D | 4 | 56 | 34 | 61 |
| 50 | A | 4 | 100 | 62 | 62 |
| | B | 4 | 100 | 53 | 53 |
| | C | 4 | 100 | 52 | 52 |
| | D | 4 | 44 | 22 | 50 |
| 51 | A | 3 | 100 | 45 | 45 |
| | B | 3 | 100 | 49 | 49 |
| | C | 3 | 100 | 46 | 46 |
| | D | 3 | 35 | 13 | 37 |
| 52 | A | 3 | 98 | 29 | 30 |
| | B | 3 | 100 | 44 | 44 |
| | C | 3 | 100 | 45 | 45 |
| | D | 3 | 25 | 8 | 32 |
| 53 | — | 3 | 694 | 694 | 100 |

In February, 1943, an attempt was made to add additional data to those recorded in table 4, but there were few plants with absolutely no evidence of at least mild symptoms. These symptoms, however, did not prevent the No. 7898 plants from maturing a crop. In the more heavily infested beds, there was some collapse in green fruit stage and many fruits fell over as they ripened, because of the weak and dried-up fruit peduncle. These latter, of course, did not produce slips, and a few scattered spots developed in the field where the plants finally died without producing ratoon suckers.

Just prior to plant crop harvest these heavily infested beds were examined to make selections of the more resistant individuals. Out of about 3500 plants in this section of the field, 14 were selected on the basis of showing no symptoms at all and 80 on the basis of showing only a very weak symptom. It was of interest that the symptom-free selections were all early-fruiting plants.

*Section 2. Single infestations to determine the sub-wilting effect, if any, of mealybug feeding.* In this experiment, five beds planted to hybrid No. 7898 and two beds to Cayenne were divided into plots 25 feet long, each containing 50 plants. Alternate plots were infested, and each infested plot was separated from another by a check noninfested plot of the same size. Infestations were again by the fruit-rind method and were made in March, April, May, and June, each month's infestation being made in a strip across the seven beds.

The results of these infestations are in table 5. Symptoms in No. 7898 were of an extremely mild type, with recovery rapid and commercial fruit maturing. Symptoms in the two Cayenne beds were typical but not the extreme type seen in the 3 infestation series mentioned previously (Table 4). Fruit weights and slip and sucker production were recorded for the plots of No. 7898. The infested Cayenne plots, while showing the typical recovery, had not recovered to fruiting by the end of the normal harvest time, and very few plants produced commercial-sized fruits even later. The only comparisons possible therefore are between infested and check plots of No. 7898 for the four separate infestations, one month apart. These records, which were taken in great detail, are summarized in table 6, which gives the analysis for significance in fruit yield as well as slip and sucker production for the four separate infestations. The March infestation resulted in the greatest reduction in all three indexes, but all four infestations reduced fruit yield, and slip and sucker production, in spite of almost negligible visual symptoms. The enhanced effect of the March infestation could be accounted for in comparison with the effects of the other three infestations by its earliness, but the results for April, May, and June are not consistent in this respect, the May infestation being out of line in fruit and slip records. The most likely explanation is to be found in the fact that the mealybugs for each infestation were naturally collected at different times and places, and the differences between infestations shown in table 6 are probably more likely

due to differences in the mealybugs rather than to plant age at time of infestation.

*Section 3. The effect on fruit and first ratoon suckers of late infestations made at monthly intervals.* In this test, two beds of Cayenne and four of No. 7898, all contiguous, were infested with single infestations made at monthly intervals from August, 1942, to October, 1942, by means of the fruit-rind method. Wilt resulting up to June, 1943, when the plant-crop fruit harvest was approaching, is recorded in table 7. There was some movement to check plants, but this was minor except in one Cayenne section.

TABLE 5.—*Wilt records from plants infested with mealybugs once. Beds 36–40, No. 7898. Beds 41–42, Cayenne*

| Bed No. | Plot No. | Date of infestation | No. of plants | Plants showing symptoms | |
|---|---|---|---|---|---|
| | | | | No. | Per cent |
| 36 | 1 | March | 50 | 14 | 28 |
| | 2 | April | 50 | 9 | 18 |
| | 3 | May | 50 | 6 | 12 |
| | 4 | June | 50 | 16 | 32 |
| 37 | 1 | March | 50 | 12 | 24 |
| | 2 | April | 50 | 10 | 20 |
| | 3 | May | 50 | 6 | 12 |
| | 4 | June | 50 | 10 | 20 |
| 38 | 1 | March | 50 | 17 | 34 |
| | 2 | April | 50 | 12 | 24 |
| | 3 | May | 50 | 4 | 8 |
| | 4 | June | 50 | 14 | 28 |
| 39 | 1 | March | 50 | 17 | 34 |
| | 2 | April | 50 | 15 | 30 |
| | 3 | May | 50 | 6 | 12 |
| | 4 | June | 50 | 23 | 46 |
| 40 | 1 | March | 50 | 21 | 42 |
| | 2 | April | 50 | 18 | 36 |
| | 3 | May | 50 | 8 | 16 |
| | 4 | June | 50 | 15 | 30 |
| 41 | 1 | March | 49 | 43 | 88 |
| | 2 | April | 50 | 47 | 94 |
| | 3 | May | 50 | 45 | 90 |
| | 4 | June | 49 | 39 | 80 |
| 42 | 1 | March | 50 | 45 | 90 |
| | 2 | April | 50 | 45 | 90 |
| | 3 | May | 50 | 44 | 88 |
| | 4 | June | 50 | 28 | 56 |

The incidence of plants with symptom expression was not decreased as much with age of plant in No. 7898 as it was in Cayenne. (Compare tables 5 and 7.) This characteristic of Cayenne is well established from previous data (1, 4, 5). Wilt in the green fruit stage of the plant's growth occurred in No. 7898 to the extent that 3.6 per cent of the total number of plants were recorded as showing symptoms.

*Section 4. The effect of removing excess ratoons on susceptibility to ratoon infestation.* In this test, following plant crop harvest of the plants

in Section 3, the surplus suckers were removed from the plants in alternate beds so that no more than two suckers were left on those plants. This particularly concerned No. 7898, which normally produces a dense mass of suckers which was thought might affect the susceptibility of the plants to infestations occurring during the ratoon growth. These infestations were made in December, 1943, and the first evidence of wilt was seen in March, 1944, with typical collapse of the ratoon Cayenne and with typical yellow areas on the distal half of the median leaves of No. 7898. By August, wilt had developed to an extreme degree.

TABLE 6.—*Effect on fruit weight and slip and sucker production of single mealybug infestations on infested No. 7898 plants which showed either no symptoms or only those of the mildest type, compared with healthy check plants. Analysis for significance between infested and check plots by "Student's" paired-plot technique*

| Index | Comparison of plots infested vs. check | Mean difference | Standard error of the difference | t value[a] |
|---|---|---|---|---|
| Fruit (g.) | Infested March | 409.4 | 48.96 | 8.362 |
| | Infested April | 273.2 | 72.52 | 3.766 |
| | Infested May | 150.6 | 40.38 | 3.730 |
| | Infested June | 283.2 | 64.80 | 4.370 |
| Slip (No.) | Infested March | 1.738 | 0.1533 | 11.337 |
| | Infested April | 0.638 | 0.2375 | 2.686 |
| | Infested May | 0.428 | 0.0739 | 5.792 |
| | Infested June | 0.720 | 0.1293 | 5.568 |
| Sucker (No.) | Infested March | 1.218 | 0.2028 | 6.006 |
| | Infested April | 0.726 | 0.2240 | 3.241 |
| | Infested May | 0.524 | 0.1568 | 3.342 |
| | Infested June | 0.504 | 0.1335 | 3.775 |

[a] The t value required for significance for odds of 19 : 1 = 2.776; for odds of 99 : 1 = 4.604.

In Cayenne, wilt was more extreme in the desuckered bed both with respect to number of plants involved and severity of symptoms. In No. 7898, symptom development was typical of that hybrid, with yellowed leaves having wilted tips, but there were scattered cases in which the whole sucker was involved as in Cayenne. Wilt was less extreme in the desuckered beds than in those in which the whole mass of suckers was left on. The increased drain of the larger number of suckers on the mother plant stump could have been the decisive factor in causing more extreme wilting among these plants.

Another curious result from this experiment was the effect the infestation in the fall of 1942 had on wilt resulting from the December, 1943, infestations. One-half of each plot in this test had been used for tests in Section 3 and had been infested in August, 1942, when the plants were about 10 months old. The other half of each plot had been a check in the previous test and had not been infested. Both halves received equal infestations in the sucker stage, but wilt following these infestations was clearly more severe in the sections which had been infested once before while the plants were young. Figure 3 illustrates this for No. 7898, but it was equally true for Cayenne.

The explanation for this phenomenon is obscure but a tenable hypothesis is that mealybug feeding affects tissues in such a manner as to injure without killing; later infestations add to the injury already sustained, with more severe wilt as a consequence of the cumulative effects.

The difference between symptom expression in the plots infested once (Table 5) and those infested several times at monthly intervals (Table 4) might also be amenable to the same explanation.

TABLE 7.—*Effect of single infestations made prior to blossom differentiation, August, 1942–October, 1942. Beds 25–26, Cayenne. Beds 27–30, No. 7898*

| Bed No. | Section No. | Date of infestation | No. of plants | No. of plants showing symptoms |
|---|---|---|---|---|
| 25 | A | August | 100 | 15 |
| | B | Check—noninfested | 100 | 3 |
| | C | September | 100 | 19 |
| | D | Check—noninfested | 99 | 0 |
| | E | October | 100 | 11 |
| 26 | A | August | 100 | 25 |
| | B | Check—noninfested | 100 | 9 |
| | C | September | 100 | 14 |
| | D | Check—noninfested | 100 | 10 |
| | E | October | 100 | 43 |
| 27 | A | August | 100 | 20 |
| | B | Check—noninfested | 100 | 0 |
| | C | September | 102 | 19 |
| | D | Check—noninfested | 100 | 0 |
| | E | October | 100 | 2 |
| 28 | A | August | 100 | 25 |
| | B | Check—noninfested | 100 | 0 |
| | C | September | 101 | 22 |
| | D | Check—noninfested | 100 | 0 |
| | E | October | 100 | 7 |
| 29 | A | August | 99 | 40 |
| | B | Check—noninfested | 100 | 2 |
| | C | September | 100 | 27 |
| | D | Check—noninfested | 100 | 4 |
| | E | October | 99 | 9 |
| 30 | A | August | 102 | 32 |
| | B | Check—noninfested | 100 | 4 |
| | C | September | 100 | 31 |
| | D | Check—noninfested | 100 | 4 |
| | E | October | 100 | 18 |

*Section 5. Uncontrolled natural infestations.* This is perhaps the most severe type of test to which plants can be subjected if the area in which they are growing is infested early in the plant's life and continues with only the normal fluctuations of an unsprayed infestation. Under these circumstances, no hybrid or variety has successfully maintained itself, although many have a considerable degree of resistance and do not have any large percentage of wilted plants until the ratoon stage. Hybrid No. 7898 has maintained itself remarkably well up to plant crop with this type of infestation, but it has a very considerable degree of wilt with severe symptoms in ratoon.

FIG. 3. *Left:* Hybrid No. 7898 susceptible to wilt following two mealybug infestations, the first prior to blossom differentiation of the first crop and the second at the ratoon sucker stage. *Right:* The same hybrid with a low incidence of wilt following a single mealybug infestation at the ratoon sucker stage.

In table 8 are the records of a number of varieties and hybrids which were selected for this test on the basis of their previous performance in resistance tests when a single infestation was used. They were planted in a small

TABLE 8.—*Wilt resulting from uncontrolled natural infestations, extending through both plant crop and ratoon, a period of about two years*

| Variety or hybrid | No. of plants | Percentage wilted | |
|---|---|---|---|
| | | Plant crop | Ratoon |
| Red Spanish | 10 | 0 | 40 |
| Cayenne | 1723 | 15 | 97 |
| Congo | 25 | 0 | 92 |
| Natal | 13 | 0 | 69 |
| Pernambuco | 6 | 0 | 17 |
| Queen | 20 | 5 | 100 |
| Ruby | 27 | 4 | 100 |
| Sarawak | 21 | 0 | 95 |
| Cayenne × Queen (No. 7889) | 19 | 0 | 29 |
| do (No. 7898) | 16 | 6 | 69 |
| do (No. 8050) | 10 | 0 | 20 |
| Cayenne × Monte Lirio (No. 8473) | 15 | 40 | 100 |
| do (No. 8705) | 16 | 6 | 93 |
| do (No. 8877) | 15 | 40 | 100 |
| Monte Lirio × Cayenne (No. 8728) | 15 | 7 | 100 |
| do (No. 8752) | 25 | 12 | 88 |
| do (No. 8817) | 16 | 0 | 100 |
| Pernambuco × Cayenne (No. 8597) | 15 | 0 | 57 |

area bounded on two sides by wild vegetation, and on the other two sides by growing pineapples. Infestation did not occur very rapidly, so after the plants were well started, a few infested fruits were scattered throughout the plot. The table presents the data for wilt in both plant crop and ratoon, and out of a very long series of varieties and hybrids included in this test, representatives have been chosen to show three types of reaction, one where a continued infestation has resulted in a high percentage of wilt in plant crop, another where a low percentage of wilt in plant crop has been followed by severe wilt in ratoon, and a third which includes all the cases in which wilt in ratoon fell below 40 per cent. The low percentage of wilt in Cayenne in plant crop undoubtedly reflects the late start that the infestation got in this plot because the percentage of wilt in that group (15 per cent) in plant crop is lower than would normally be expected. It should be noted that although several of the hybrids showed no wilt up to inflorescence stage, none of these survived the continued infestation in ratoon, although there was some difference in the severity of the symptoms which developed.

### SYMPTOM EXPRESSION IN NO. 7898 WHEN INFESTED WITH MEALYBUGS FROM THAT HYBRID

The toxin hypothesis of mealybug wilt has as a natural corollary the expectation that the nutrition of the mealybug on the plant from which it is moved might affect its toxicity to the plant to which it is moved. The significance of this in breeding programs for resistance is very great. Tests have naturally been conducted with bugs reared on the commercial variety, Cayenne, but in the event of a new hybrid being substituted for Cayenne, mealybugs would then move, under field conditions, within plantings of that new hybrid.

The first evidence that a variety could react differently to mealybugs which had been reared on that variety than to mealybugs from a different variety was available several years ago in a small test with Cayenne and Queen (the two parents of hybrid No. 7898). The first transfer using Cayenne bugs resulted in 60 per cent wilt in the Cayenne to Cayenne sequence and 10 per cent in the Cayenne to Queen sequence. When the mealybugs were transferred from these plants to a second series of test plants some months later, the Cayenne to Cayenne sequence gave 86 per cent wilt and the Queen to Queen 68 per cent.

In the large planting of No. 7898 in which most of the experiments with that hybrid thus far recorded were conducted, a small section at one end of the planting was left unsprayed. This became infested in due course but there were few plants showing any sort of symptom by plant crop, and the ratoon growth was vigorous. A new planting of No. 7898 was made next to this infested ratoon with the expectation that it would become infested with bugs from the latter. This occurred and the resulting effect on the young plants was significant. The symptoms resulting from this "hybrid to hybrid" infestation were typical of wilt in Cayenne. The entire plant

was involved, color changes were not limited to a few leaves, and there was considerable downward drooping of the plant typical of 3rd-stage wilt (Fig. 4). Many of these plants, however, recovered by fruiting time, but in some cases dried peduncles resulted in drooping fruit.

A field experiment was then devised to determine the effect of the variety to variety sequence of mealybug feeding. In this test, mealybugs from plantation Cayenne and from No. 7898 were used for cross infestations by the fruit rind method. These infestations were sprayed out after 26 days.

FIG. 4. Hybrid No. 7898 plant going into typical 3rd-stage wilt, as a result of natural infestation of mealybugs from an older planting of the same hybrid. The plant had a lighter color than nearby plants, the dry leaf tip, and the downward drooping of the median leaves.

The results of this test were significant in two respects. Symptoms on No. 7898 which were infested with mealybugs from No. 7898 developed 3rd-stage wilt typical of Cayenne. This confirmed the results in the field plot mentioned above. The effect of No. 7898 bugs on Cayenne, however, was unexpected. Wilt following this infestation had a shorter period for development of symptoms which progressed rapidly with a complete collapse of all the plants resulting. Mealybugs from Cayenne to Cayenne also wilted all the plants in that plot but development of symptoms was slow and plant collapse was not complete (Fig. 5).

This increase in toxicity to a susceptible variety, of mealybugs which are grown on a resistant variety, is probably unique. The nearest analogous phenomenon is perhaps the increased virulence to a susceptible corn variety

of *Phytomonas stewarti* grown in resistant varieties (6, 7), but the explanation on the basis of the segregation of genetic strains of the bacteria does not hold in the case of mealybugs because the change in toxicity has occurred in mealybugs following a single transfer to the resistant variety.[4]

### TESTS OF HYBRID POPULATIONS HAVING THE RESISTANT HYBRID NO. 7898 AS ONE PARENT

Following evidence of resistance in the hybrid No. 7898 and in the varieties Queen and Pernambuco, individual plants of a hybrid population, Lot 1760, produced by crossing No. 7898 with No. 8597, the latter an $F_1$ Cayenne × Pernambuco selected clone, were planted as clones in the fall of 1941. These clones, with an average of 3.6 plants each, were infested with mealybugs by the fruit rind method early in 1942.

FIG. 5. Cayenne plants (right) infested with mealybugs from the resistant hybrid No. 7898; the same variety (left) infested with mealybugs from Smooth Cayenne.

Records of wilt occurring in these clones were made in August, 1942, when the plants were about 10 months old and again in July, 1943, at the age of about 21 months. Pineapple plants usually produce a mature fruit in 18 to 20 months and most of these hybrids had fruited or were in fruit at the time of the second wilt record in July, 1943.

Table 9 shows segregation of these clones into wilting and nonwilting groups, with susceptibility to wilt again appearing as dominant. The figures of 232 wilting to 65 nonwilting clones approximates a monogenic $F_2$ ratio.

The phenomenon of recovery from wilt mentioned as characteristic of

[4] Unpublished data.

the No. 7898 hybrid is also shown in some of the clones of Lot 1760 derived from No. 7898 as one parent. Table 9 lists 104 such clones which showed wilt following infestation with mealybugs when the plants were 10 months old but which were listed as symptom-free by fruiting time in 1943.

TABLE 9.—*Record of wilting in clones grown from single $F_1$ plants produced by crossing hybrid No. 7898 with No. 8597 (Lot 1760)*

| | |
|---|---|
| Total number of clones | 297 |
| Number showing no wilt in 1942 | 91 |
| Number showing no wilt in 1943 | 169 |
| Number showing no wilt in both years | 65 |
| Number wilted in 1942 and recovered in 1943 | 104 |

The clones of Lot 1760 which were susceptible to wilt were discarded at the end of the 1943 test. Clones appearing to be resistant were replanted in the fall of 1943 and again infested with mealybugs early in 1944 with the following records obtained in July, 1945: 69 clones wilting, 7 clones showing delayed wilt, 10 clones showing no wilt.

The tabular data presented here from numerous infested varieties and hybrids demonstrate that very rarely do all the plants of an infested susceptible group have wilt symptoms. Because of this characteristic some of the small clones may fail to show wilt in one test but will do so in another. However, the larger the clone the more readily are wilt symptoms detected in that clone if it is a susceptible one.

Throughout these experiments clones were classified as susceptible when they contained a single wilting plant. As was pointed out, the first clonal generation of Lot 1760 hybrids contained an average of 3.6 plants per clone. Some of these classified as resistant in the 1942 and 1943 records showed wilt in the second vegetative generation test in 1945. The number of plants per clone in the second generation (14.6 plants per clone) was large enough to give increased confidence in the segregation of resistant from susceptible clones.

Both the parents of Lot 1760 were derived from varieties (Queen and Pernambuco) which were more resistant to wilt than the Cayenne variety. A number of the clones discarded after the 1943 tests were similar to the No. 7898 parent in degree of resistance and ability to recover from wilt. Selections, however, were directed toward obtaining a higher degree of resistance than was found in the parental varieties.

## SUMMARY

Pineapple varieties collected from widely separated geographical areas have been tested for resistance to mealybug wilt. From these tests it was determined that Cayenne, the commercial variety in Hawaii, is one of the most susceptible.

Tests with variety hybrids, particularly those in which Cayenne was used as one of the parents, indicate that susceptibility is a dominant character.

Detailed tests have been made with a Cayenne-Queen hybrid (No. 7898) for which resistance was first demonstrated, in comparison with Cayenne, in commercial plantings under conditions of natural infestation.

This hybrid shows only mild leaf symptoms under conditions of mealybug infestation which will produce severe symptoms in Cayenne. Under more severe conditions of infestation, namely, repeated infestations at monthly intervals, and continuous infestation, symptoms are severe and approximate those seen in Cayenne. Mealybugs grown on No. 7898 induce more typical symptoms on No. 7898 than do mealybugs from Cayenne. Symptoms on Cayenne appear more rapidly and develop to much greater severity when mealybugs from No. 7898 are used.

The susceptibility of Cayenne diminishes markedly with age of plant but that of hybrid No. 7898 does not appear to be affected by this factor to the same degree. Infestations of both Cayenne and No. 7898 made just before the differentiation of the inflorescence, increase the susceptibility of the same plants to infestations made a year later on the ratoon suckers.

Sub-wilting effects in No. 7898 plants which have shown only a mild leaf symptom, express themselves in reduced fruit size and reduced number of vegetative slips and suckers.

Studies with hybrids in which No. 7898 is one of the parents show evidence of segregation of the resistant character.

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# NOTES ON A BROWN PIGMENT AND OTHER UNUSUAL CHARACTERS IN CULTURES OF BACTERIUM MARGINATUM[1]

LUCIA McCULLOCH

(Accepted for publication February 19, 1947)

*Bacterium marginatum* L.McC., when grown on beef media, has no particularly distinctive characters, either macroscopic or microscopic; but on Thaxter's potato agar[2] a brown pigment and several unusual forms are produced so consistently and readily that this medium becomes the best means of identification of the organism.

The unusual color and forms characteristic of this important gladiolus pathogen are here briefly described with the hope that someone will be interested in carrying on biochemical investigations of the nature of the pigment and why it is deposited, almost entirely, in the bacteria and certain kinds of associated spheres. The "starch crystals" previously described[3] also deserve further investigation.

Detailed notes, photographs, drawings, microscope slides, and dry specimens are deposited in Mycological Collections, U. S. Bureau of Plant Industry, Soils, and Agricultural Engineering. Beltsville, Md.

Cultures of *Bacterium marginatum* may be obtained from the American Type Culture Collection, 3900 Reservoir Road, Washington 7, D. C.

*Bacterium marginatum* on Thaxter's potato agar[4] first produces a white, thick and soft growth later collapsing into a thin brownish layer. Cultures 1 to 4 days old have, along with the numerous bacteria, thousands of small hyaline spheres, mostly mere points in size; but many are 4 to 7 $\mu$ and others up to 20 $\mu$ in diameter. Material mounted for microscopic examination is opaque because of light refracted from the numerous spheres. A drop of 1 per cent NaOH placed at the edge of the cover glass causes the almost instantaneous disappearance of the spheres, leaving a transparent mount of bacteria. These spheres form as soon as growth is established and persist only as long as the medium is acid in reaction. During growth the medium, which is acid, increases in acidity, the pH reaching 3.8 to 4.0. As the peak of growth passes, the acidity declines until, with completed growth, the pH value is 8.0 to 8.6.

[1] McCulloch, Lucia. A bacterial disease of gladiolus corms and leaves. Jour. Agr. Res. [U.S.] 29: 159–177. 1924.

[2] Prepared according to formula given in Bitancourt, A. A., and Anna E. Jenkins. New discoveries of Myriangiales in the Americas. Proc. Eighth Amer. Scient. Congress, Washington, D. C., 1940. 3 (Biological Sciences): 154. 1942.

[3] McCulloch, Lucia. Starch-like radiate crystals produced by Bacterium marginatum in starch media. Jour. Agr. Res. [U.S.] 39: 495–501. 1929.

[4] All descriptions and illustrations are from Thaxter's potato-agar cultures unless otherwise noted.

These hyaline spheres stain readily, but seldom take the same color as the bacteria. In osmic acid they become black and brittle. Boiling distorts them slightly or produces a granular appearance. Dried on a glass slide and held in a blue flame they show no visible change in 20 seconds, but with 30 seconds of flaming they disappear. Pressure on the cover glass flattens some of the larger spheres and continued pressure causes dissolution and disappearance.

As the hyaline spheres disappear, a brown color begins to develop in the growth and reaches its maximum in 7 to 10 days. This brown color varies from pale amber to "tawny russet" and "raw umber" of Ridgway.[5] Examination shows that the color is not uniformly diffused but occurs in definite areas, usually as a fine network of branching lines (Fig. 1, E). Microscopic examination shows that the brown areas are composed of closely packed bacteria and spheres, translucent, golden brown to opaque dark brown, with an inconspicuous number of normal hyaline bacteria. Some of the brown bacteria are normal in size and shape but many are abnormal (Fig. 1, B).

Because of their number and size the brown spheres (Fig. 1, A) are more noticeable than the brown bacteria. Spheres 0.5 to 0.8 $\mu$ predominate, those 5.0 to 25.0 $\mu$ are abundant, a few are 40.0 to 45.0 $\mu$, and one of 60.0 $\mu$ was observed. With age, if the culture medium remains sufficiently moist, the brown spheres, particularly the larger ones, develop a hyaline wall (Fig. 1, C, a). Soaking in cold or hot water usually causes the wall to appear. Pressure and movement of the cover glass flattens some of the larger spheres temporarily, and occasionally a sphere breaks into irregular fragments or into several smaller spheres. The wall, if present, usually breaks and separates from the brown center (Fig. 1, D). In moist Van Tieghem cells, kept under observation for long periods, some spheres developed the hyaline wall and also showed a further development in the breaking up of the wall into a number of small, hyaline spheres which eventually disappeared (Fig. 1, C, b–g).

The origin of the brown spheres is unknown. Possibly they may be the results of deposition of pigment in a dead bacterial cell which may then enlarge to form a sphere, and a capsule which is not pigmented swells to form the hyaline wall. (*Bacterium marginatum* has very definite capsules.) Secondly a granule from a bacterial cell may become pigmented and swell to form a sphere, but without a hyaline wall.

This theory would account for the fact that there seem to be two types of spheres, the walled or capsulated spheres, usually large, translucent, and apparently mere empty shells, and spheres lacking walls, dark or even opaque, and, when broken, divided into smaller but still opaque parts.

The brown spheres and bacteria persist indefinitely, even in old, dry cultures. They are extremely resistant to dry heat, to steam (15 lb. pres-

[5] Ridgway, Robert. Color standards and nomenclature. 43 pp. 53 color plates. Washington, D. C. 1912.

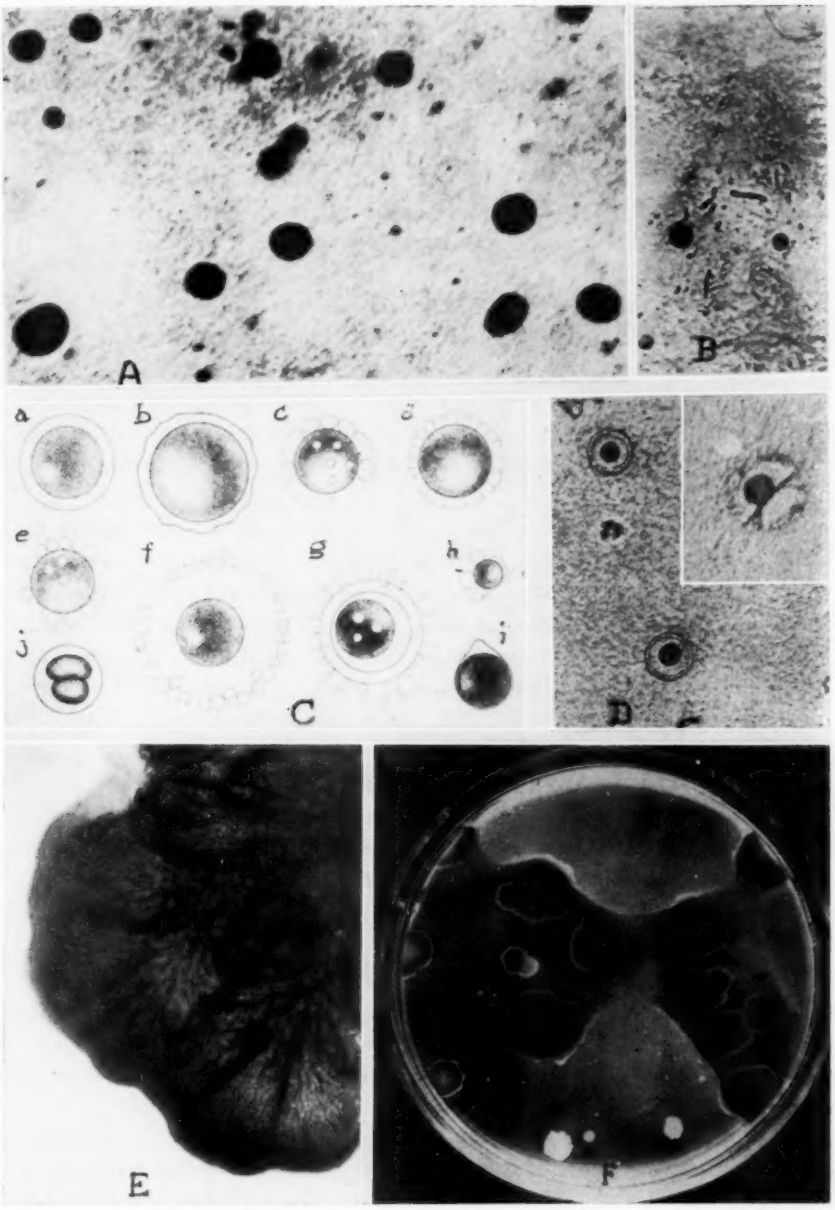


FIG. 1. A. Brown spheres. Not stained. ×600. B. Brown bacteria and three small brown spheres. Not stained. ×750 approx. C. Brown spheres with hyaline walls in various stages of dissolution. Not stained. (Drawings by L. C. C. Krieger from camera lucida drawings and measurements from Van Tieghem cell cultures by L. McC. The measurements given are the total diameters of the sphere and its wall.) *a*. Hyaline wall, uniform in thickness. Common type in cultures. 14 μ. *b*. Wall irregularly thick. Early stage of breaking up. 18 μ. *c* and *d*. Later stages in wall disintegration. 14 μ and 14.8 μ. *e*. Same sphere as *c* but 11 days later. 14 μ. *f*. Same sphere as *a* but 9 days later. 23 μ. *g*. Double wall and projections. 23 μ. *h*. Several attached hyaline spheres. 29 μ. *i*. Example of irregular wall. 9 μ. *j*. Two spheres in one wall. 11 μ. D. Brown spheres, wall of one broken by pressure. ×400. E. Section of a colony showing the brown lines. ×6. F. Plate culture showing clearing of the agar. ×½.

sure), to boiling water, and to alcohols; and they are attacked only slightly, if at all, by acids and alkalies. The only means so far discovered for destruction of these brown bodies, is subjecting them to a blue flame for 2 to 3 minutes.

Slight bleaching was caused by concentrated glacial acetic acid, concentrated carbolic acid, 5 to 50 per cent HCl, 4 to 25 per cent NaOH, aqua regia, and strong ammonia.

Slight darkening was caused by concentrated HCl, 50 per cent NaOH, and 50 per cent $H_2SO_4$.

Extreme darkening, even to blackening, was caused by concentrated $H_2SO_4$, 4 per cent osmic acid, and a saturated solution of picric acid.

These changes usually required exposure to the reagents for several days and sometimes for weeks. Acids in which brown bodies have been soaked become more or less brown even when there is no visible loss of color in the spheres. The addition of water to the browned acid produces a brown precipitate.

The originally rather cloudy potato agar becomes more nearly transparent as the bacterial growth develops. In plate cultures the clearing begins at the margin of the colony and spreads until all of the agar is cleared (Fig. 1, F). In slanted agar tubes the clearing begins at the top of the slant and, if the agar is deeply slanted, continues to the base of the tubes. The cleared agar is negative for reducing sugars and the starch is apparently not lessened.

Imbedded in the cleared agar, sphero-crystals composed of radiating, needle-like parts are abundant. These are 15 to 35 $\mu$ in diameter, a few being somewhat larger. These crystals are formed only in media containing starch and the fact that they become blue when treated with iodine solutions suggests that they may be some form of starch. They are referred to by the writer as "starch crystals."

Mycelium-like forms (Fig. 2, A, B, C) develop occasionally in the potato agar and abundantly in 2 to 3 per cent solutions of dextrose or galactose. These forms, 2 to 200 $\mu$ long, are mostly slender with smooth continuous walls while others are wholly or partly like chains of bacteria. They have considerable motility in young cultures. Most of these filamentous forms are hyaline but in agar cultures a few are yellow to brown.

For the production of hyaline spheres, of brown spheres and bacteria, and "starch crystals," Thaxter's medium seems to be the most generally favorable and reliable. Best results were obtained with pH values of 5.0 to 5.5. Other media were tried in an effort to determine which substances are necessary for the production of the different forms. The experiments are far from comprehensive but they do demonstrate that neither potato nor starch are necessary for the development of the brown forms. Typical brown bacteria and brown spheres were produced in synthetic media. Starch is essential for the development of "starch crystals." The mycelium-like forms develop best in 2 to 3 per cent solutions of dextrose or galactose.

Brown forms have been found, but not abundantly, in the pellicles of the dextrose-solution cultures.

Numerous other species of bacteria causing plant diseases were grown parallel with *Bacterium marginatum*, but none of these over showed a trace of the pigment of forms so common in *Bact. marginatum*.

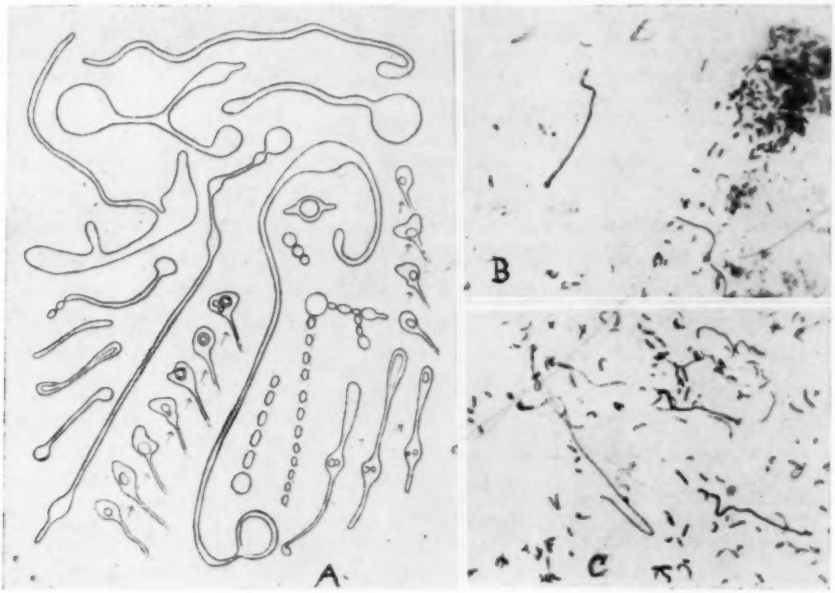


FIG. 2. A. Mycelium-like forms of *Bacterium marginatum*. Camera lucida sketches. Approx. 800×. B, C. Photographs of the mycelium-like forms, stained and much shrunken.

## SUMMARY

*Bacterium marginatum* L. McC., when grown on suitable media, produces in addition to the usual bacterial cells great numbers of small, evanescent, hyaline spheres. These soon disappear and are followed by less numerous but larger brown spheres which are permanent and extremely (practically entirely) resistant to the many chemical reagents tried. Brown bacteria also occur. The brown pigment is almost entirely confined to the bacteria and the spheres.

In the cleared parts of the agar cultures, below the bacterial growth, sphero-crystals 5 to 35 $\mu$ are abundantly produced. Since these crystals are formed only in media containing starch and become blue when treated with iodine solutions, it is thought they may be some form of starch.

In solutions of dextrose or galactose (2 to 3 per cent) numerous, motile myceliumlike forms develop.

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# TEST TUBE DILUTION TECHNIQUE FOR USE WITH THE SLIDE-GERMINATION METHOD OF EVALUATING PROTECTANT FUNGICIDES[1]

THE AMERICAN PHYTOPATHOLOGICAL SOCIETY. COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS

(Accepted for publication February 2, 1947)

The test tube dilution technique is eminently suited for preliminary slide-germination tests of new chemicals as fungicides. It is more simple than the horizontal sprayer or settling tower technique, is more rapid than the latter and no elaborate apparatus is required. For water-soluble materials it is the most precise technique, while for chemicals giving ready and stable suspensions it is reasonably accurate. It is not accurate for difficultly suspendible chemicals and cannot be used for laboratory "rain" tests of tenacity. The technique as described here is an elaboration of one previously developed (3, 4). It should be used in conjunction with the standard slide-germination method (1) in which there is given the necessary background information and relevant detail of supporting techniques.

## APPARATUS

Test tubes without lips, 150 × 18 mm. (1 for each dilution of each chemical and each fungus). Transfer pipettes—5 ml. (1 for each chemical)—2 ml. (1 for each chemical and each fungus). Two-ml. pipettes graduated in 0.5-ml. units (1 for each fungus). Test tube wire support racks to hold 40 tubes each (at least 1 for every 10 chemicals and every fungus). Graduate cylinders—50 ml. (1 per chemical). Erlenmeyer flasks—250 ml. (1 per chemical). Other general apparatus as ordinarily required for the slide-germination method.

## PREPARATION OF DILUTION

Unless otherwise stated, rapid and approximate dilution methods are used, since the errors thus introduced ordinarily do not equal or exceed day to day variation (3, 4). Weigh out 0.5 gram of the chemical to be tested, dissolve or suspend in 50 ml. of distilled water, measured from a 50-cc. graduate cylinder and placed in a 250-ml. Erlenmeyer flask. After thorough shaking, withdraw 2 ml. by means of the 2-ml. transfer pipette and place in a test tube, being careful not to run down inner sides of tubes. Place test tube in wire support rack and label tube with wax pencil to identify compound. Repeat for each fungus using a different rack.

*Dose Ratio of 10.* Withdraw 5 ml. from the stock one per cent (10,000 p.p.m.) preparation by means of a 5-ml. pipette, discard remainder from flask, rinse rapidly from wash bottle, and add the 5-ml. sample. Add 45 ml. of distilled water, shake thoroughly. Rinse the 2-ml. pipette in this new dilution, discard rinsing solution then withdraw 2-ml. samples as before, and place in test tubes which in turn are placed beside the first tube in the rack. In case of 4 dilutions or less, run tubes crosswise of rack, if more than 4, place lengthwise. No label is needed as the compound and dilution are identified by position. Continue to dilute as above until a series of 4 or 5 test-tube dilutions are obtained, giving 10,000, 1000, 100, 10, and 1 p.p.m. of chemical. The first or last in this series frequently may be omitted, depending on circumstances.

*Graduate Cylinder Alternate.* After first concentration is made and the necessary 2-ml. samples placed in test tubes, add 5 ml. to a 50-ml. graduate cylinder and add distilled water to make 50 ml. Agitate thoroughly by pouring back and forth into the 250-ml. Erlenmeyer flask or beaker. Withdraw necessary 2-ml. samples from flask for

[1] Reprints may be obtained at 10 cents each from the Committee Chairman, Boyce Thompson Institute, Yonkers 3, New York.

the test tubes. Add 5 ml. to the graduate cylinder which has been rinsed, and make up to 50 ml. with distilled water. Continue dilutions in same manner until all desired are obtained. The 2-ml. pipette is kept in the test tube for later use.

The dilution series for the second chemical is prepared as for the first, except, of course, fresh glassware is used throughout. The first test tube containing the most concentrated chemical is likewise labeled and placed in the wire support rack beside the corresponding one for the first chemical. Additional dilutions and tubes are placed side by side with the first series, so that in one axis of the wire support rack there is a series of the same chemical and in the other a series of the same dilution.

*Dose Ratio of* $\sqrt{10}$. This series usually follows preliminary results obtained with a dose ratio of 10. Start with lowest dilution giving no germination. For second dilution, place in 50-ml. graduate 16 ml. of first dilution, and 34 ml. of distilled water. Agitate thoroughly by pouring into 250-ml. Erlenmeyer flask as above and withdraw necessary 2-ml. samples for test tubes. Add 16 ml. from flask to graduate, and 34 ml. of distilled water, agitate as before, withdraw 2-ml. samples as before. Continue dilution series as far as desired.

*More Accurate Dilution.* In case of tests with soluble materials where high precision is desired or a very low dosage is used, it will be necessary to employ more orthodox methods of dilution. Here volumetric flasks in conjunction with pipettes or burettes should be used and the whole series prepared and placed in a series of Erlenmeyer flasks before the 2-ml. aliquots are withdrawn.

## SPORE GERMINATION STIMULANT

In many cases it is necessary to add a spore stimulant to insure a high and relatively stable percentage of germination in the checks. For this purpose various agents have been employed, *e.g.*, orange juice (4), potassium citrate plus sucrose (5), extracts from dried potato-dextrose agar (6), and coenzyme R (2). The stimulant used should be stated.

*Orange Juice.* Orange juice stimulant is prepared by filtering the juice of several good quality oranges through cheesecloth, followed by filter paper, and finally through a large Berkefeld cylinder, type W, under vacuum. The resulting clear filtrate is diluted ten-fold with distilled water and 10-ml. portions placed in small corked shell vials which are stored at below freezing temperatures. This constitutes 10 per cent ultra filtered orange juice. When needed for use, dilute contents of one vial to 100 ml. which gives the desired concentration to add to the spore suspension (see below); the final concentration in which the spores are exposed for germination will be 0.1 per cent. Enough stock orange juice may be prepared and frozen to last several years. New batches may be expected to differ slightly in potency but may be standardized against the old if desired.

*Other Stimulants.* Details regarding the use of other stimulants may be seen in the original papers cited above.

## ADDING SPORES AND STIMULANT

Spores of the desired fungus are suspended in water according to the standard method (1). A concentration of 500,000 spores per ml. should be attained by means of a counting cell. To an equal volume of the spore suspension there is added an equal volume of the spore stimulant solution of a concentration 10 times that finally desired. If no stimulant is added the initial spore suspension concentration should be reduced to 250,000 per ml. The mixture is well stirred by blowing through a 2-ml. pipette graduated in 0.5 ml. units; 0.5-ml. samples of the spore suspension stimulant mixture are then pipetted into each test tube containing the 2 ml. of diluted chemical. Before withdrawing each pipetteful the mixture must be thoroughly stirred by blowing. One pipetteful will suffice for the 4 test tubes arranged crosswise of the support rack, and the place between rows of "spores added" and "not added" may be simply indicated by moving each tube in turn one space sidewise, or by changing the slope of the tubes or by laying a pencil or pipette between the rows.

## PLACING DROPS ON SLIDES

Four or five glass slides are placed in each moist chamber in a horizontal position to the operator and two drops from each test tube of spores and chemical are pipetted side by side on to the left-hand side of a glass slide. For this purpose the 2-ml. transfer pipettes employed to place the most dilute chemical in the test tube are again used. However, fresh pipettes will be needed for each extra set of fungi and chemicals. Hence when pipetting work from the most dilute chemical to the most concentrated and from the top or furthermost slide to the nearest, the second fungus is placed on the right-hand

side so that similar dilutions are placed on the same slide, and only one chemical is in a single moist chamber. If a given chemical is volatile and toxic and the range of dilutions is wide, the compound may be overrated because of the volatilization from concentrated drops and resulting condensation in dilute drops. This will be corrected in a second test where a lower dose ratio and smaller range of dilutions is used.

When the chambers are filled, the "tops" are placed in position, the water seal added and germination counts made 20–24 hours later, according to the standard method.

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# STANDARDIZED SPRAY NOMENCLATURE[1]

THE AMERICAN PHYTOPATHOLOGICAL SOCIETY
COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS

(Accepted for publication February 19, 1947)

The standardized spray nomenclature for deciduous fruit trees, here presented, has been attained through the cooperation of plant pathologists, entomologists, and horticulturists representing the various pertinent regions of the United States. The terms employed are believed to express the best general and current usage.

## DOSAGE

All spray materials are to be expressed in standard units of weight (avoirdupois) or liquid measure (U. S. System) sufficient to make 100 gallons of spray mixture. Examples:

"*Bordeaux mixture 8–8–100*," or 8 pounds of copper sulfate, 8 pounds of lime (hydrated or burnt) with sufficient water to make 100 gallons of spray mixture.

"*Liquid lime sulfur 2–100*," or 2 gallons of liquid lime sulfur (32° B = 1.2823 sp. gr.) in sufficient water to make 100 gallons of spray fluid.

"*Lead arsenate 3–100*," or 3 pounds of lead arsenate with sufficient water to make 100 gallons of spray mixture.

All dust materials to be expressed in standard units of weight (avoirdupois) sufficient to make up 100 pounds of dust. Examples:

*20–80 copper-lime dust*, or 20 pounds of copper sulfate mixed intimately with 80 pounds of hydrated lime.

*20–80 basic copper arsenate-sulfur dust*, or 20 pounds of basic copper arsenate mixed intimately with 80 pounds of 300 mesh or fine elemental sulfur or any equivalent of a proprietary conditioned sulfur.

*1 per cent Rotenone dust*, or 1 pound Rotenone (extract or in crushed roots) mixed intimately with talc, bentonite, chalk, tobacco dust, walnut flour, wheat flour, as the case may be, to make 100 pounds of dust mixture.

## PART I—APPLE AND PEAR SPRAY TERMS

*Dormant.*—From time the leaves have fallen in the fall until the buds show silver tips in the spring is regarded as dormant spray period. Application should never be made when temperatures approach freezing, but preferably at temperatures about 40° F.

*Pre-bloom.*—From time blossom buds begin to show the silver tip stage until first blossoms open.

*Delayed dormant.*—When green tips begin to show until leaves of blossom buds are one-fourth to one-half inch exposed.

*Pre-pink.*—When leaves are separated, leaving individual flower buds exposed in a tight cluster. Often referred to as "early closed cluster," "early pre-pink," "early pre-bloom," "pre-bloom," "late closed cluster," or "beginning of closed cluster." Temperature dominates the passage of one stage to another and for spraying purposes all stages should be considered as "pre-pink" since it is rarely possible to time this spray to the separate stages.

*Pink.*—When the flower buds have separated in the cluster, exposing the pedicel and the petals of each flower bud but not the stamens and pistils. Often referred to as "open cluster," "pre-pink," or "full pink."

*Bloom.*—When the flower buds have opened, exposing the stamens and pistils in from 50 to 75 per cent of the flowers. Full bloom corresponds to having 90 per cent or more of flowers open.

*Petal fall.*—When at least 75 per cent of the petals have fallen and before the calyx lobes have closed, often referred to as "calyx."

*Cover sprays.*—When the calyx lobes have closed and the fertilized fruits begin to increase in size. The sequence of the different cover sprays would follow the particular conditions existing in the different fruit sections and the application should follow closely the recommendations of the local authorities or be closely aligned to the spray schedules outlined in each state. These local recommendations are usually arrived at after years of study and the industry is usually informed by telephone, radio, and by letter of any

[1] Reprints may be obtained at 10 cents each from the Committee Chairman, Boyce Thompson Institute, Yonkers 3, New York.

last minute changes to fit the conditions of the season. The cover sprays are usually designated according to their sequence: first, second, third, etc., or first brood and second brood sprays when referring to infestation of codling moth.

PART 2—PEACH, PLUM, APRICOT, AND ALMOND SPRAY TERMS

*Dormant.*—From time the leaves have fallen in the fall until the buds begin to swell the following spring is regarded as the dormant spray period.

*Early pink bud.*—When buds have emerged or burst but petals are only partially exposed; known as "red bud" in apricot and "bud swell" in almond.

*Pink bud.*—When buds begin distinctly to show the full color of the petals. Known as "late red bud" in apricot, "popcorn" in almond, and "puff ball" in peach.

*Bloom.*—When 10 per cent to 75 per cent of the blossoms are fully open.

*Petal fall.*—When 75 per cent of the petals have fallen.

*Cover sprays.*—When the young and growing fruits have been completely separated from their enveloping calyces. First cover known as "shuck fall" spray for peach. Cover sprays usually not needed in apricot and almond.

PART 3—CHERRY SPRAY TERMS

*Dormant.*—From the time the leaves have fallen in the fall until the buds begin to swell the following spring is regarded as the dormant spray season.

*Pre-bloom.*—When most of the buds show color of petals prior to full opening.

*Petal fall.*—When 75 per cent of the petals have fallen.

*Cover sprays.*—When the "shucks" (calyces) have fallen completely from the fruits, usually a week to 10 days after petal fall and including a spray 10 days before harvest and one following harvest.

PART 4—QUINCE SPRAY TERMS

*Dormant.*—From the time the leaves have fallen until the buds swell the following spring is regarded as the dormant season.

*Bloom.*—When the flowers are 90 per cent or more in full bloom.

*Cover sprays.*—When the calyx lobes are closed and the young fruits are expanding.

# REPORT OF THE TWENTY-EIGHTH ANNUAL MEETING OF THE PACIFIC DIVISION OF THE AMERICAN PHYTOPATHOLOGICAL SOCIETY

The 28th annual meeting of the Pacific Division of The American Phytopathological Society was held in Berkeley, California, June 24 through June 27, with the Division of Plant Pathology of the University of California as host. A record attendance was had with 70 members and 10 non-members present, representing Arizona, British Columbia, California, Nevada, Oregon, Utah, Wisconsin, and Washington. The program consisted of four sessions of scientific papers, two business meetings, one day of field trips, and a 3-session Fruit Tree Virus Symposium. At one of the business meetings it was voted that the Secretary-Treasurer serve as Councilor with the A.A.A.S., Pacific Division.

The following officers were elected for 1947:

*President:* C. E. YARWOOD
*Vice President:* L. C. COCHRAN
*Secretary-Treasurer:* GEORGE W. FISCHER (2 years)
*Councilor:* MAX W. GARDNER

Following are the abstracts submitted:

*Perennial Nightshade, a Host for Corynebacterium michiganense.* R. C. BAINES. Perennial nightshade plants (*Solanum douglasii*) affected with stem cankers were found in a tomato field in southern California in 1944. Cultures of a bacterium similar to *Corynebacterium michiganense* were isolated and produced typical infection on nightshade and tomato plants. An isolate from tomato was pathogenic on tomato but not on nightshade. *C. michiganense* was found to overwinter on the perennial nightshade.

*Heterosporium Disease of Nasturtium and Its Control.* KENNETH F. BAKER. The extensive nasturtium seed fields in coastal California often sustain severe damage in mid-summer from an undescribed Heterosporium leaf spot. This production disease is largely limited to California. The fungus, internally carried in seed up to 47 per cent, infects the stem from the old hypogeally germinated seed and after the plant breaks over at soil level, sporulates abundantly. Infection of the leaves occurs through hydathodes and insect injuries, and, by harvest, plants often are defoliated. In the moist environment beneath plants the fungus penetrates pericarps and seeds. Green seed cannot be infected, even by wounding, until the pericarp becomes senescent, when rapid invasion and sporulation occur. The pathogen survives at least 3 years in seed. Infected seeds and volunteer or carryover plants supply initial field inoculum. Excellent control was achieved, with only 3.3 per cent average germination loss, by treating seed 30 minutes in water at 51.7° C. after a 1-hour soak in cool water. The disease was eliminated in extensive tests when treated seeds were planted in areas free of infected plants, and reduced when grown near infested areas by delayed infection. Seeds are easily indexed when held moist in glasshouse flats to determine infection.

*Transmission of Rhizoctonia solani in Pepper Seed and Its Prevention.* KENNETH F. BAKER. Pepper seedlings in field seedbeds and glasshouse flats usually sustain severe losses from Rhizoctonia pre- and post-emergence damping-off in southern California. This soil fungus developed in pasteurized soil planted with infected seed. Internal seed transmission was demonstrated by planting on moistened, sterile black peat in Petri dishes and by sectioning. Pepper fruits, particularly large types, frequently rest on the ground in seed fields and much Rhizoctonia "soil rot" occurs before the delayed harvest. The fungus grows into the seed from the funiculus in the micropyle-radical region. Maceration and fermentation in cleaning favor further infection. The fungus also is carried in small bits of fruit tissue. Up to 0.3 per cent of commercial seed is infected. This infection is important in (a) contaminating pasteurized or noninfested soil and (b) perpetuating strains causing damping-off, wire-stem, and fruit decay. Only certain strains infect the fruit and therefore become seed-borne. Treatment of the seed in water at 51.7° C. for 30 minutes eliminated the fungus without germination loss in extensive tests during three years. Nontreated seed produced 42.0 per cent fewer marketable plants in pasteurized soil than did treated seed. The treatment also controls similar infections of eggplant and zinnia seed.

*Observations on the Root Endophyte, Rhizophagus, in Culture.* J. T. BARRETT. There are many records of occurrence and study of the Rhizophagus type of endophytic fungi in roots of many plants. Of these none has reported success in establishing the

organism in culture although slight growth has resulted in a few cases. After several unsuccessful attempts the writer has obtained a culture of *Rhizophagus* from the root of garden pea. This was accomplished by placing a small piece of boiled hemp seed directly on a few hyphae attached to a piece of infected root in water. After a few days new growth was apparent. The piece of seed bearing the new growth was transferred to a Petri dish containing sterile tap water with a fresh half of boiled hemp seed submerged in the center. Within ten days mycelium bearing numerous typical vesicles had covered the bottom of the dish. Subsequently the fungus was established on water agar plus a small amount of water from autoclaved hemp seed and on other media. For the first time one species or strain of this interesting cosmopolitan root endophyte is made available for a better taxonomic and biological study.

*Myrothecium Leaf Spot and Canker of Gardenia.* J. T. BARRETT and DORIS ANN HARDMAN. In a previous report of this disease, in 1941, it was suggested that the causal organism was a new member of the family Tuberculariaceae. Subsequently the pathogen was identified as *Myrothecium roridum* Tode and our work furnishes the first and only report of its occurrence in California. The fungus is known now to attack also gardenia cuttings in rooting beds under high humidity and high temperature conditions. Successful infection of tomato plants with and without previous injury and of tomato fruit with needle punctures confirm in part work previously done in England. The disease is not widespread and apparently is easily prevented by using proper culture conditions.

*Controlling Fruit Spoilage in Dates.* DONALD E. BLISS. Disastrous crop losses of dates occur in California about one year in five. Moist weather during August and September is conducive to fungus spoilage (*Aspergillus niger* V. Tiegh., *Alternaria citri* E. and P. em. Bliss and Fawcett, etc.) and to insect infestation. A search was begun in 1940 for an effective protectant fungicide that would be easily removed from ripened dates and nontoxic to man. Among 11 fungicidal treatments tested in the field, significant differences in fungus spoilage of Deglet Noor dates were obtained as follows: in 1943, sulphur dust; in 1944, sulphur and a dust containing 5 per cent Fermate in sulphur; and in 1945 (a year of severe spoilage), Fermate-sulphur mixture and a dust containing 10 per cent Yellow Cuprocide in sulphur. Whereas in 1945 untreated dates had 53.18 per cent fungus spoilage, those treated with Fermate-sulphur mixture had only 11.11 per cent. Fruit quality and palatability were not reduced. Date mites, *Paratetranychus simplex* (Banks), were controlled by the treatment, and relatively few dried fruit beetles, *Carpophilus hemipterus* L., were found. Residues of ferric dimethyl dithiocarbamate (Fermate) on treated dates were reduced to 4.90 p.p.m., and those of sulphur, to 11.82 p.p.m. by the Nussbaum cleaning method. Fermate-sulphur mixture is being tested commercially.

*Sparse Leaf of Italian Prune.* EARLE C. BLODGETT. A disease affecting several trees in one orchard in southwestern Idaho was observed first in 1942. The affected trees were characterized by, (1) a delay in blooming and leafing of 10 days or more, (2) sparse foliage confined largely to the periphery of the trees giving them an umbrella shape, and (3) marked reduction in fruitfulness although the fruit is of good size. The trees are not dwarfed. There is no good evidence of orchard spread. Leaf symptoms are expressed as small leaves with irregular margins and mild diffuse mottling. In general appearance some of the leaves are similar to those of cherry crinkle leaf. Bud inoculation tests indicate that sparse leaf is bud perpetuated but to date there is no proof of transmission.

*Studies on Tip Burn of Sugar Beet and Lettuce.* EUBANKS CARSNER. Sugar-beet tip burn develops on etiolated leaves in total darkness under conditions of high humidity. Recovery of plants so affected occurs in total darkness under relatively low humidity. Tip burn is more severe under low light intensity if the humidity is high. Lettuce tip burn is directly comparable to sugar-beet tip burn in regard to the relationships of light intensity, nitrogen nutrition, humidity, vigor of growth, age of plants, and recovery. The responses to environmental influences, both in showing symptoms and in recovery, are more rapid in lettuce than in sugar beet.

*Phyllody of Common Beans, a Graft-Transmissible Disease.* B. F. DANA. Scions from phylloid inflorescences of naturally infected bean plants were cleft-grafted on greenhouse-grown plants of Red Kidney, Bountiful, Asgrow Stringless Green Pod, and U. S. No. 5. Phyllody occurred in nine of ten inoculated plants, and scions from their phylloid inflorescences transmitted phyllody to 11 of 20 plants of Logan, Red Kidney, Bayo, Bountiful, and U. S. No. 5. No transmission was obtained by inserting wedges of tissue from phylloid plants into stems of 32 inoculates. Phyllody has occurred locally in East-

ern Oregon on varieties of common beans that are resistant to curly top as well as on plants of susceptible bean varieties that escaped infection. A similar disease, big bud, occurs on tomato; and what appears to be phyllody has occurred on soybean, lima bean, alfalfa and squash. Phyllody, apparently induced by aster-yellows virus, also has appeared on aster, scabiosa, delphinium, California poppy, chrysanthemum, marigold, cosmos, and carrot, which are known to be hosts of aster-yellows virus. This latter fact suggests that the simultaneous occurrence of phyllody symptoms on common bean, lima bean, and soybean, tomato, alfalfa, and squash may be due to infection by one or more strains of the aster-yellows virus.

*The Spreading Factor in Plant Tissues.* J. DUFRENOY. Hyaluronidase, the "spreading factor" affecting animal tissues, is prevalent in invasive bacteria, in the poisonous secretions of snakes or insects, and in the sperms of mammals. Plant pathogens spreading rapidly from the infection site, are apt to induce in cells at a distance a dissociation of the cytological constituents into nucleoproteins and fatty droplets, and an active secretion of phosphorylated compounds into the intercellular spaces. Pathogens unable to induce such changes in normal tissues may cause a spreading lesion only when and where a mechanical, physical, or chemical shock already had caused a breakdown of cell constituents. For instance, *Ovulinia azaleae* spots petals bruised by beating rains, by pushing hyphae between epidermal and subepidermal cells, which, as long as they survive, release compounds to the intercellular spaces. Pollen tubes, growing in the tissues of the style, behave as efficient parasites, in promoting the secretion of compounds from nearby living cells.

*Reversible Sensitization of Plant Tissues Following Heat Treatment.* J. DUFRENOY and H. S. REED. Heating plant tissues at 50–52° C., before inoculating with spores of pathogens, may lower the resistance, as observed by Edgerton *et al.* in stalks of sugar cane which received a hot-water treatment before being inoculated with conidia of the red-rot fungus. Conversely, heating after inoculation inactivates conidia so that they fail to develop a red-rot lesion; more important still, canes affected by chlorotic streak may be freed of the virus by the hot-water treatment. Heating at 50–52° C. causes a reversible swelling of mitochondria and thus a temporary dispersion of the complexes of nucleoproteins and lipids, now recognized as the cytological site of activity of dehydrogenases, whereas the copper proteins, acting as polyphenol-oxidases, are scarcely affected by temperatures below 60° C.: therefore, heating above 50° but below 60° C. causes a decompensation of respiration, as dehydrogenases are no longer apt to make hydrogen available as an acceptor to molecular oxygen, activated by oxidases. Heat treatment, properly applied, induces a shock from which cells recover, as mitochondria regain their normal appearance of rods or threads, staining sharply with reagents for phosphorus.

*Some Studies on Curly-Top of Potatoes.* N. J. GIDDINGS. Seedling potato plants of the variety Earlaine have been inoculated in the greenhouse with curly-top virus, and 43 per cent of the plants became infected. Infection was secured with 8 of the 10 virus strains used and with other virus selections which had not been designated as strains. The percentage of plants infected was greatest among those plants inoculated with the more virulent strains but one plant was infected by strain 7 which produces only very mild symptoms on sugar beet. Many of the infected plants did not yield any tubers but the tubers from 18 of them were saved and plants grown from them were tested. The 28 tubers from 16 seedling plants gave 26 plants in which the presence of curly-top virus was demonstrated and two in which it was not, while 25 tubers from 2 plants were all negative. Third generation plants were not tested. In commercial potato fields tests from plants with symptoms suspected of being curly top have been negative in every instance thus far. Ninety-one such tests have been made and include suspected material from Oregon, northern California, Bakersfield, Shafter, and Perris, California. Extensive inoculation experiments with different curly-top-virus strains have been made using White Rose, Katahdin, and Russet Burbank potato varieties. Very heavy inoculations have resulted in a small percentage of infection in each of the potato varieties both in the greenhouse and in the field. Field inoculations in replicated plots have thus far given no evidence of significant yield differences between the inoculated and the noninoculated plots.

*The Comparative Value of Certain Organic and Inorganic Sulphur Compounds in the Control of Botrytis Blight of Tulips.* C. J. GOULD. Bordeaux and certain other mixtures or compounds containing copper have been recommended for the control of tulip blight (caused by *Botrytis tulipae*), but severe injury has often resulted from the use of these sprays. Since 1942 in field tests with 24 different sprays or dusts, certain organic sulphur compounds consistently have given excellent blight control with little or no burning. In order of decreasing control the sulphur compounds may be roughly arranged as follows: Fermate (ferric dimethyldithiocarbamate), Zerlate (zinc dimethyldithiocarbamate), Ter-

san (tetramethyl thiuram disulphide), Fermate-sulphur dust, zinc ethylene bisdithiocarbamate, Dithane (disodium ethylene bisdithiocarbamate), potassium resin polysulphide, and sulphur dust. Phenothiazone was promising in one year's test. The best material in each of the 5-year's tests was Fermate (2 lb./100 gal.).

*Sodium Arsenite, a Promising Control of Dead-Arm of Grapes.* WM. B. HEWITT. Sodium arsenite solution containing an equivalent of 3 lb. arsenic trioxide per 100 gal. water was sprayed on grape vines, variety Olivette blanche, infected with *Cryptosporella viticola* Shear during dormancy, not earlier than 3 weeks after pruning. Three blocks of vines, 6 rows each, averaging 43 shoots per vine, sprayed February 11, 1944, had an average of 4.9, 4.9, and 5.1 diseased shoots per vine; corresponding nonsprayed blocks had 27.0, 25.5, and 22.6 diseased shoots per vine. Similar blocks of vines sprayed in 1945 had very little disease. Shoot and leaf infections were light in 1946; however, in 3 blocks of vines sprayed on March 14 there were 2.6, 18.7, and 7.3 per cent of the vines with one or more diseased shoots and in corresponding nonsprayed plots 46.6, 27.5, and 52.6 per cent of the vines similarly diseased. The extrusion of spores from pycnidia on diseased spurs collected at random from the 1944 plots was as follows: 1–10 per cent of the pycnidia on 38 out of 138 sprayed spurs and 60–85 per cent of the pycnidia on 93 out of 106 nonsprayed spurs extruded spore horns.

*Hydrogen Fluoride Injury to Prune Trees in Washington.* FOLKE JOHNSON, V. L. MILLER, and D. F. ALLMENDINGER. Hydrogen fluoride, given off in smoke from aluminum factories, was found to produce considerable injury to Italian prune orchards in western Washington. The injury began as a marginal scorch of the leaves with necrotic areas appearing in the center of the lamina. This injury was followed by a severe leaf drop in mid-summer. The fluorine content of 52 samples of oven-dried prune leaves collected from localities where aluminum factories were located varied from 18 to 1400 p.p.m. In contrast, five leaf samples from a factory-free area had a fluorine content which varied between 6 and 15 p.p.m. Leaves with only a slight injury had a lower fluorine content than those with severe leaf spotting and marginal scorch, and the necrotic areas had a higher fluorine content than the green tissue. Similar symptoms were produced in peaches, plums, apples, pears, English walnut, Italian prunes, strawberries, and raspberries when subjected for eight days to a concentration of 5 p.p.m. hydrogen fluoride by weight, in a sealed chamber. Only doubtful symptoms were noticed in filberts and sour cherries at this concentration. Injury to prune leaves was also produced after treatment with several fluorine compounds used as sprays.

*Fungicides for the Control of Brown Rot of Citrus.* L. J. KLOTZ and G. A. ZENTMYER. Because of the aggrevation of HCN fumigation injury to citrus trees by prior coverage with copper sprays, pathologists have for many years sought for a non-copper spray material that would be effective against Phytophthora brown rot of citrus fruit. The search has been intensified during the past two seasons because in some localities citrus trees have been directly and severely damaged by copper sprays even in the absence of HCN fumigation. Many fungicides, including the new organic spray materials, are being tested. Results thus far show that no satisfactory substitute has been found. However, among those tested and found worthy of further study are: disodium ethylene bisdithiocarbamate with zinc sulphate and lime, tetrachloroquinone, zinc dimethyl dithio carbamate and 2,3-dichloro-naphthoquinone. While in laboratory experiments these materials have shown promise approaching the efficacy of Bordeaux mixture, in the grove they have failed to weather satisfactorily and to protect fruit from decay by the brown-rot fungi.

*Tissue Relationships of Certain Dodders to Some Host Plants Used in Virus Disease Studies.* C. F. LACKEY. Some dodders have been found useful in the transmission of certain virus diseases. Tissue relationships of three dodders have been studied on some of the host plants used in virus disease studies. The species used were *Cuscuta californica* Choisy, a very small dodder, *C. subinclusa* Dur. and Hilg., a larger one, and *C. americana* L., the largest of the three. All grow fairly well on sugar beets and tobacco, *Nicotiana tabacum*. *C. americana* grew best on *N. glauca*. There its haustoria do not pass through the xylem ring but make good connections with the external phloem. On *N. tabacum* it grows through the xylem ring into the pith, making only limited connections with the internal phloem. *C. californica* and *C. subinclusa* make but few connections with the vascular tissue of the cucumber and so do not grow vigorously on it. *C. subinclusa* makes no vascular connections with certain squash varieties and so does not grow on them. *C. californica* grows well on tomato, making good vascular connections. *C. americana* does not grow vigorously on tomato. Hypertrophy of cortex cells is induced which interferes somewhat with vascular connections between the dodder haustoria and the host. *C. subinclusa* will not establish itself on tomato, because mere contact of its

haustoria with tomato stems produces great hypertrophy of the cortex cells in the region of contact. Further growth of haustoria to apparently inhibited and enlarged host cells prevent haustoria reaching the vascular tissue. Lack of vascular connections between dodder and host would prevent transmission of phloem-limited viruses such as the curly-top virus.

*Localized Chemical Applications to the Soil and Their Effects upon Root Rots of Beans and Peas.* LYSLE D. LEACH and WILLIAM C. SNYDER. The prevention of bean root rot (*Rhizoctonia solani, Fusarium solani* f. *phaseoli, Thielaviopsis basicola*) and pea root rot (*R. solani, F. solani* f. *pisi, Ascochyta spp.*), hitherto largely dependent only upon cultural practices (crop rotations and cover cropping), has been attempted by chemical soil treatments. Applications of chemicals in the row when seeding, similar to the formaldehyde-drip treatment against onion smut, have been made in artificially infested soils in the greenhouse and in naturally infested soils in the field. Of the materials so far used, 25 per cent disodium ethylene bisdithiocarbamate (Dithane D14) has proven most effective. When applied at the rate of 1 gal. per acre, or 2 lb. of the dry powder (Dithane A10) per acre, marked reductions in the degree of root-rot infection were obtained. It has not been determined whether the action is directly fungicidal or fungistatic, or indirect through its effect upon the host. Materials of this kind applied locally as a soil treatment in the planted row, on or with the seed, seem to offer a promising approach to the control of root-disease complexes of a variety of crops.

*The Effect of Terminal Smut Galls on Yield and Seed Grade of Detasseled Hybrid Corn.* J. D. MENZIES and C. O. STANBERRY. Galls of common corn smut frequently occur at the top of corn stalks that have been detasseled for hybrid seed production. These galls result in yield reduction which varies directly with the size of gall. In this study of 864 smutted plants, galls under 2 inches in diameter reduced the yield 8.8 per cent, while galls of 2 to 3 inches in diameter and over 3 inches in diameter reduced yields 14.1 and 40.1 per cent, respectively. Yield reduction resulted both from smaller ears and fewer ears per stalk. This effect was accompanied by smaller-sized kernels and a slight increase in cull grade. The average yield loss per plant in marketable grain due to these terminal smut infections was calculated to be 22 per cent. Smut did not affect the germinability of the seed.

*Root Rot of Condiment Sage.* JOHN T. MIDDLETON. A disease affecting the root, crown, and stem of mature sage, *Salvia officinalis*, was observed in the Imperial Valley of California in a planting grown for seed. Diseased plants are stunted, wilted, and the leaves ash grey-green in color; badly diseased plants do not produce inflorescences and usually die. Infected fibrous roots are discolored, the cortex water-soaked and flaccid. Longitudinally oriented, sunken, dark brown to black, lesions that may girdle the root, frequently occur in the larger secondary and primary roots. Infection usually progresses upwards, causing cortical necrosis and discoloration of the outer portion of the stem stele; lesions may extend 2 to 4 inches above the soil. The disease is most conspicuous where plant beds are low, irrigation water stands, and drainage is poor. Isolations from diseased material collected in February, 1946, yielded *Phytophthora parasitica*, while those made from March collections yielded *P. parasitica* and *Pythium aphanidermatum*; only *P. aphanidermatum* was secured from May samples. The pathogenicity of each of the two fungi to sage has been demonstrated by artificial inoculation. Inoculations with mixed cultures neither depressed nor accelerated disease development. Symptoms produced by either pure or mixed cultures of the fungi are similar to symptoms on naturally infected plants.

*The Production of Ascochyta-Free Pea Seed in Southern California.* JOHN T. MIDDLETON and W. C. SNYDER. Pea seed devoid of *Ascochyta* has been produced from both naturally and artificially infected seed when grown in the Imperial and Temecula Valleys of southern California. Naturally infected Alaska, naturally and artificially infected Little Marvel, and artificially infected No. 76 seed have been used. Peas at Temecula were grown during the summer rain-free period, while those in Imperial, near Brawley and Calipatria, were grown during the winter and late spring. The average rainfall at Brawley for the 5-month fall period, August through December, totals 1.19 inches, while that for the corresponding spring period, November through March, totals 1.64 inches; the annual rainfall is 2.43 inches. At no time throughout the three-year period of study have aerial symptoms of Ascochyta blight been observed in any of the plantings. Seed harvested from plants grown from infected seed have been proved to be free of *Ascochyta*. Other seed-borne diseases have likewise not been observed on aerial portions of the plant. Plants grown from healthy pea seed sown in land cropped to peas for the past 23 years had neither below- nor above-ground symptoms of Ascochyta blight.

*Sieve-Tube Necrosis in Orange Trees Affected by Quick Decline During the Spring Season.* HENRY SCHNEIDER. In the spring of 1946 the necrosis of sieve tubes of the sour orange stock of sweet orange trees, at and below the bud union, was similar to that found in the winter. In the process of necrosis, callus forms on the sieve plates; then the sieve tubes and companion cells are crushed by the expansion of the adjacent parenchyma cells, which are occasionally divided and usually cleared of fat globules and starch. Wound gum was not found. The following stages of necrosis have been found: (1) Those in which necrosis was present a short distance below the bud union. Contrary to normal obliteration, which sometimes appeared earlier in sour than in sweet orange, necrosis usually occurred first in the youngest or medium-aged sieve tubes in trees having no top symptoms. (2) Those in which the older, outer sweet orange sieve tubes above the union had also become necrotic, apparently because of the girdling effect. (3) More advanced stages in which the cambium had produced new phloem. In these cases a narrow cylinder of functioning phloem was usually present below the union in the sour orange stock and a wider one above the union; but at the union the sieve tubes were callused or necrotic. In 3 trees out of 10 that were in the equilibrium stage when sampled in June, there were a few functioning sieve tubes near the cambium at the union.

*Newly Discovered Leafhopper Vectors of California Aster-Yellows Virus.* HENRY H. P. SEVERIN. Nineteen newly discovered leafhopper species, and three species and one biological race previously reported, are vectors of the virus as follows: Short-winged and long-winged (biological race) aster leafhoppers, *Macrosteles divisus* (Uhler). Phlepsius group: *Paraphlepsius apertinus* (Osborn and Lathrop), *Texananus lathropi* Baker, *T. pergradus* DeLong, *T. spatulatus* Van Duzee, *T. oregonus* Ball, *T. latipex* DeLong. Thamnotettix group: *Idiodonus heidemani* (Ball), *I. kirkaldyi* (Ball), geminate leafhopper, *Colladonus geminatus* (Van Duzee), mountain leafhopper, *C. montanus* (Van Duzee), *C. commissus* (Van Duzee), *C. flavocapitatus* (Van Duzee), *Friscanus intricatus* (Ball), *F. rupinatus*, *Acinopterus angulatus* Lawson. *Gyponana hasta* DeLong, the first reported vector in the subfamily Gyponinae. *Cloanthanus irroratus* (Van Duzee), *C. dubius* (Van Duzee). *Euscelis maculipennis* DeLong and Davidson, *Fieberiella florii* (Stal). *Chlorotettix similis* DeLong. Twenty-two leafhopper species and one biological race belonging to twelve genera have been demonstrated to be vectors of the virus.

*Transmission of Virus of Pierce's Grapevine Disease by Spittle Insects.* HENRY H. P. SEVERIN. Four species and six varieties belonging to three genera are vectors of the virus as follows: *Aphrophora angulata* Ball, *A. permutata* Uhler, *Clastoptera brunnea* Ball, *Philaenus leucophthalmus* var. *leucophthalmus* (Linnaeus), *P. leucophthalmus* var. *pallidus* (Zetterstedt), *P. leucophthalmus* var. *fabricii* Van Duzee, *P. leucophthalmus* var. *marginellus* (Fabricius), *P. leucophthalmus* var. *spumarius* (Fallen), and *P. leucophthalmus* var. *impressus* DeLong.

*Studies of a Bud Failure Condition in Almond Trees.* GILBERT L. STOUT and E. E. WILSON. A disorder in almond trees, the chief character of which is failure of many buds to live and develop into new growth, occurs in many orchards in north central California. The bud failure often results in a peculiar type of twig and limb development and in disorderly arrangement of twigs and branches, suggesting the term "crazy top." Other symptoms include dying of twigs, delay in blooming, sparsity of foliage, and, in the Peerless variety, brown necrotic areas on twigs produced during the previous season. These areas later become rough and cracked. In both Nonpareil and Peerless trees, bands of rough bark up to a foot in width develop on older wood. Buds and scions from affected Nonpareil and Peerless trees failed to transmit the disorder to normal Nonpareil, Peerless, and Drake trees within five years, but a high percentage of buds and scions from Nonpareil and Peerless perpetuated it. Scions from healthy Nonpareil placed in affected Nonpareil have remained normal after eight years. A bud failure of Drake similar in some respects to that in Nonpareil and Peerless has been transmitted with scions to Nonpareil within two years. This suggests that the disorder in Drake may be different from that in Nonpareil and Peerless.

*An Electron Microscope Study of Two Strains of Potato X Virus.* WILLIAM N. TAKAHASHI and T. E. RAWLINS. Larson's potato virulent ring-spot and potato latent-mottle viruses were studied with the electron microscope. They were found to be indistinguishable in form and size. A high proportion of the particles have lengths between 500 and 600 mμ. The particles lack the rigidity characteristic of tobacco-mosaic virus and are often curled into various forms. The similarity in appearance of the two strains of potato X virus is in agreement with the idea that mutations are frequently not accompanied by detectable changes in the appearance of the virus particle.

*An Electron Microscope Study of Mutation in Tobacco-Mosaic Virus.* WILLIAM N. TAKAHASHI and T. E. RAWLINS. Common tobacco-mosaic virus and a yellow strain isolated from it were studied with the electron microscope. The yellow strain was found to be indistinguishable in size and form from the parent strain. It is concluded that mutation in this case was not accompanied by a modification of the virus particle sufficiently great to be detected by the electron microscope.

*The Use of Triethylene Glycol Vapor as an Aid in the Control of Air-Borne Contaminants.* W. J. VIRGIN and JEAN C. MALOIT. Considerable trouble is often experienced in pathological laboratories with air-borne contaminants when doing various types of cultural work. Steam is often employed but it has definite disadvantages. The use of triethylene glycol vapor has proved very effective in eliminating air-borne contaminants and has no uncomfortable effect on the worker. A beaker containing 100 cc. of triethylene glycol placed on a low flame of a Bunsen burner will effectively fume a room of 7,000 cu. ft. in about 20 min. and will use approximately 40 to 50 cc. of liquid. Smaller rooms will take proportionately less. The liquid becomes discolored on heating but this has no effect on its ability to vaporize. The vapor rises to the ceiling and gradually settles down to the floor. It appears that as the vapor settles it carries down with it most of the organisms that may be floating around in the air. Exposing Petri dishes containing agar for 5 min. before and after fuming showed that there was considerable reduction in the number of air-borne contaminants after fuming as compared to before fuming.

*Copper Carbonate for Chestnut Root Disease.* WILLIS W. WAGENER and JAMES W. KIMMEY. In the summer of 1942 there appeared in an 80-year-old planting of European chestnut a crown rot, apparently caused by *Phytophthora*, but attempts to isolate the causal organism have been unsuccessful. In May, 1943, twelve trees were treated by a method reported by Landaluze in Spain. The soil was excavated from around the bases of the trees, records were made of dead areas present, and the cleaned bark was painted with a suspension of copper carbonate in water to which ½ lb. per gal. of casein glue had been added as an adherent. The soil was then refilled around the trees. Three trees, 70 per cent or more girdled by the disease when treated, later succumbed or became completely girdled; three others showed small extensions of killings; and the remaining six suffered no further injury. While the treatment did not fully arrest the disease, it showed evidences of some beneficial effect. On this basis and that of the promising results from somewhat similar treatments reported from Spain, it is believed that additional trials of the method here are warranted where this type of crown rot becomes active.

*The Vertical Dispersion of Spores in the Air Near the Ground by Winds of Low Velocity.* E. E. WILSON. During overcast periods when thermal turbulence was weak and probably absent the comparatively large (33 μ) spores of *Lycopodium* were carried upward when released 3 ft. from the ground into air moving 0.6 to 5.4 m.p.h. Judging from the ratio of the aerial density of the spores above the level of release to the density at the level of release, vertical dispersion per unit of downwind distance was greatest at the lowest wind velocity, and diminished rapidly as the velocity increased. A similar trend was apparent on sunny days when some thermal turbulence probably existed. Ratios pertaining to dispersion downward from the 3-ft. level likewise diminished as air movement increased. On an overcast morning and with wind velocity 1.5 m.p.h., spores were carried to earth from a height of 3 ft. within a distance of about 12 feet. The number intercepted by the ground increased from this distance to about 20 feet and declined thereafter.

*Influence of Host, Fungicide, and Pathogen on the Utility of a Spreader in Fungicide Tests.* C. E. YARWOOD. In greenhouse tests the addition of 0.05 per cent phthalic gylcerol alkyl resin spreader to bluestone spray decreased its injury on bean, cucumber, and mustard foliage but increased its injury on pea foliage; increased its eradicant (therapeutic) action for bean powdery mildew and cucumber powdery mildew and for bean rust and sunflower rust; increased the protective action of Bordeaux and other insoluble coppers for bean rust, bean powdery mildew, snapdragon rust, onion downy mildew, and cucumber downy mildew; increased the protective value of lime-sulphur for onion downy mildew and snapdragon downy mildew; but decreased the protective value of lime-sulphur for bean powdery mildew, and cucumber powdery mildew, and of Fermate (ferric dimethyldithiocarbamate) for snapdragon rust. The ratio of the LD95 of fungicide sprays without spreader to the LD95 of the same fungicide with spreader ranged from 1: 4 with lime-sulphur as a protective spray for cucumber downy mildew to 455: 1 with Bordeaux as a protective spray for snapdragon rust.

*Yield from Potato Giant Hill.* C. E. YARWOOD. In the San Juan Bautista district of California the dying of Netted Gem potato plants at 70 to 100 days from planting has

been associated with infection by *Verticillium alboatrum*, *Rhizoctonia solani*, *Phytophthora infestans*, and *Alternaria solani*, the first being most important. Giant Hill vines occurring naturally in plantings of certified seed have lived up to 135 days and have been less severely infected with or injured by these fungi. Giant Hill selections yielded less than normal vines up to 90 days from planting, but yielded 35 to 63 per cent more weight of tubers at 120 to 135 days from planting in 1942 to 1945. At Half Moon Bay, Giant Hill plants yielded 30 per cent more than normal at 106 days from planting in 1944, but normal plants yielded more than Giant Hill at Stockton and Berkeley in 1945. Giant Hill tubers were larger, had more overgrowths, deeper eyes, and had a tendency to be spindle-shaped, but at maturity were similar in cooking quality to normal tubers. Strains of Giant Hill differed with respect to longevity of vines, length of vines, susceptibility to early blight, yield of tubers, and shape of tubers.

*Black Canker of Cherry.* S. M. ZELLER, J. A. MILBRATH, and J. R. KIENHOLZ. Black Canker of Napoleon sweet cherry has been found in Polk, Union, Wasco, and Washington counties, Oregon. Cankers start in one-year-old twigs, first as slightly swollen areas in which the bark splits lengthwise. These areas grow into rough black cankers the ultimate size of which is more or less determined by the size of the affected branch. Some infected trees are very severely cankered, while others may have few cankers. No abnormal fruit or leaf symptoms have been observed. Many attempts to isolate a causal organism have failed, but transmission of the disease was brought about by graft inoculation after 2-year incubation.

*The Rôle of Microorganisms in Avocado Tree Decline.* GEORGE A. ZENTMYER and L. J. KLOTZ. Avocado decline has been recognized occasionally in California for many years but only recently has assumed considerable proportions. Poor drainage of the soil, occurring either on very heavy soils or on soils with an impervious layer near the surface, generally initiates the trouble. A specific biological factor or factors may also be involved. Avocado seedlings have made greater (3–4 times) and more vigorous growth in sterilized (autoclaving or chloropicrin) soil than in nonsterile soil from "decline" areas. At least two factors appear to be involved in decline: waterlogging and *Phytophthora cinnamomi*. When present, *P. cinnamomi* may accelerate decline, but decline occurrence is not dependent on the presence of this fungus alone. Injury will occur from waterlogging periods of 10–14 days or more in soils which have been sterilized with chloropicrin or by autoclaving. Inoculation of soil with *P. cinnamomi* has resulted in injury to the root system even when excess water was not present. Toxic products formed by bacteria and other microorganisms under anaerobic conditions, including butyric acid, nitrite, and $H_2S$, also play a part in decline. Such products are increased and consequently root injury is greater in soils in which the carbohydrate content has been increased (addition of sugar).

## PHYTOPATHOLOGICAL NOTES

*Injury to Plants by Minute Amounts of 2,4-Dichlorophenoxyacetic Acid.*[1]—Many new weed killers containing salts or esters of 2,4-dichlorophenoxyacetic acid, apparently effective for the purpose intended, are now available. It appears, however, that they must be used with extreme care to avoid injury to valuable plants. Unintentional injury may be caused to sensitive plants in at least 4 ways: namely, from toxic residues left in spray or dust-mixing equipment, not removable by the usual methods of cleaning or washing; vapors from equipment and containers; absorption by roots; and drift of spray or vapors by air currents. These conclusions are based on observations of accidental injury and on laboratory tests.

Following use with 0.1 per cent ethyl ester of 2,4-D, a galvanized iron hand sprayer was washed 3 times with hot water and soap, then rinsed several times with hot water. A few weeks later rose, zinnia, and phlox were sprayed with an insecticide from this sprayer. Some time later the new rose shoots were spindling and the leaflets were narrow, deeply toothed, curled, and often mottled. The young leaves from the terminal buds of phlox were narrow and mottled and had irregular margins. Numerous shoots that developed in the axils of the abnormal phlox leaves often were of jack-in-the-pulpit type. The apical leaves of zinnia were very narrow and yellow and usually died. The leaves of shoots that developed in the axils of uninjured leaves of zinnia appeared normal.

In an experimental greenhouse, potted tomato and cabbage plants were injured by what appeared to be a volatile material in the air. The upper leaflets were narrow and rolled and the leaves bent downward along the curved stem. These abnormalities resembled the early symptoms of 2,4-D injury on rose and phlox; therefore, it was suspected that injury to tomato and cabbage was due to fumes of 2,4-D from a wheelbarrow sprayer placed in the greenhouse after a 0.1 per cent butyl 2,4-D had been used in it. One lot of potted tomato plants from this greenhouse, transplanted to the field 2 days after the sprayer had been placed in the house, appeared normal when set in the field; but symptoms of injury appeared later. Another lot of potted tomato plants from this greenhouse, transplanted to the field before the sprayer had been placed in the house, developed normally without injury of any kind.

To test whether fumes of butyl 2,4-D injured tomato and cabbage, and to eliminate other gases as a cause of injury, the sprayer was placed in an open insectary. A tomato and a pepper plant, placed in the open tank, developed leaf rolling and drooping in 12 hours. The sprayer was then moved to another building. Tomato, cabbage, pepper, zinnia, petunia, tobacco, and *Nicotiana rustica* developed leaf rolling and drooping after 1

[1] The investigation reported in this paper is part of a project of the Kentucky Agricultural Experiment Station and is published by the permission of the Director.

to 2 days when set in a closed room at some distance from the open spray tank.

In addition to leaf rolling and drooping, tomato, tobacco, and petunia plants, after several days' exposure to fumes of 2,4-D developed raised, white galls on the upper swollen stems and petioles. Root initials formed on these galls in a moist atmosphere. In tomato gradually the epidermis died and peeled off, causing the upper leaves to wither. In some cases, in a moist atmosphere, guttation was prominent on many leaves and sometimes water droplets oozed from the stems as the epidermis cracked. Several tomato plants injured by fumes of 2,4-D produced seedless fruits. Numerous shoots formed at the base of the tobacco plants.

An attempt was made to remove toxic residues of butyl 2,4-D from the wheelbarrow sprayer by successive washings with a saturated solution of trisodium phosphate, 1 part concentrated hydrochloric acid to 5 parts of water, and strong soap and water. After each washing, zinnia, petunia, cabbage, and *Nicotiana rustica* plants, exposed to fumes from the sprayer, were injured.

A 0.1-per cent emulsion of butyl 2,4-D was sprayed on experimental grass plots in March. In mid-May, in a nearby fence row and door yard, there were abnormal secondary shoots of rose, grape, hackberry, mulberry, locust, oak, redbud, mock orange, Virginia creeper, and pokeweed. The early growth on all of these species was normal. Most of the injured shoots were some distance above the ground and several feet from where the chemical was applied. On grapevines, 50 feet away, and on roses, at least 25 feet away, there were marked malformations and mottling. It is believed that vapors of 2,4-D, rather than drift of the chemical at spraying time or washing of the material to the roots and later absorption, were responsible for injury to these plants both because of the time elapsing between application and injury and because of the distance of the injured plants from the point of application. It appeared unlikely that 2,4-D could have washed to the roots in this area, although vapor may have drifted at the time of application. These observations indicate that this method of weed control in gardens and lawns must be used with care to prevent drift of the chemical or vapors to valuable plants, especially if the highly volatile compounds are used.

Laboratory tests with the ethyl and butyl esters of 2,4-D indicate that toxic residues of both esters may remain in metal spray equipment after successive washings in hot water and soap, and strong alkali. Two washings of metal sprayers with acetone apparently removed most of both esters; however, some injury still occurred to zinnia, petunia, and *Nicotiana rustica* when these plants were placed under a bell jar with one of the sprayers and to tomato plants sprayed with water from the sprayers.

Both esters caused injury to tomato, cabbage, petunia, *Nicotiana rustica*, and tobacco when either concentrated solutions or dilute emulsions were placed under bell jars with these plants. These tests indicate that it is diffi-

cult to remove toxic residues of the esters of 2,4-D from spray equipment and that volatile products from equipment and containers may injure sensitive plants in closed rooms or greenhouses. It is doubtful whether power equipment could be washed sufficiently by the usual methods to make it safe for other uses.

Three washings with tap water appeared sufficient to remove toxic residues of both the ammonium and sodium salts of 2,4-D from metal sprayers. There appeared to be no toxic volatile products from either the dry chemicals or solutions of the ammonium or sodium salts of 2,4-D because sensitive plants exposed to the dry salts and solutions for 5 days under bell jars were not injured.—E. M. JOHNSON, Kentucky Agricultural Experiment Station.

*Gaseous Sterilization of Biological Materials for Use as Culture Media.*—The ordinary procedure of sterilizing biological materials by heat has the disadvantage that it sometimes alters drastically the physical-chemical nature of the substance being sterilized. In addition there are frequently inconveniences attendant upon heat sterilization.

In order to circumvent these disadvantages as far as possible and to approach more nearly the need for sterile natural media for the purposes of isolating and culturing fungi,[1] both ethylene oxide and propylene oxide have been tried as sterilizing fumigants in place of heat. Their successful use over a 2-year period has prompted this note.

Although the use of these fumigants as insecticides is well known, it was the work of Whelton *et al.*[2] which suggested to the writers that such chemicals might be used for the preparation of sterile natural media. These investigators were confronted with the comparable problem of sterilizing dehydrated fruits in a manner which would leave the product in as natural a condition as possible, free from the caramelization and unnatural odors and flavors that attend heat pasteurization, and free from toxic residues which may result from sterilization.

The material to be sterilized, whether it be alfalfa hay, pea pods, dry bean straw, wheat seed, youngberry fruit, fresh carrot, dehydrated fruits and vegetables, etc., insects, or even soil mixtures, is placed in a container such as a fruit jar and if very dry is moistened slightly by lightly atomizing it with water or by placing a piece of wet blotting paper in the container. The fumigant (propylene oxide) is measured approximately in a graduated cylinder or pipette and introduced into the jar at the rate of about 1 cc. per liter capacity of the container. The cover is immediately screwed down over the mouth of the jar with a gasket to make it air-tight. The jar is shaken and put aside overnight or longer, at room temperature. The lid may then be loosened and the fumigant allowed to escape, after which the material is ready for use. Media for multiplication of inocula may be prepared in a

[1] Snyder, William C., and H. N. Hansen. Advantages of natural media and environments in the culture of fungi. Phytopath. **37**: (in press). 1947.

[2] Whelton, Rita, H. J. Phaff, and E. M. Mrak. Control of microbiological food spoilage by fumigation with epoxides. Food Industries **18**: 23–25; 318–320. 1946.

similar way. The dosage indicated here is probably excessive and no doubt could be cut down considerably for most materials. Ethylene oxide because of its low boiling point cannot be handled satisfactorily in this way at room temperature without special equipment.

In the preparation of natural media for culturing organisms the treated material may be combined directly with liquid or solid media, such as agar, or used alone. For example, in the preparation of a Petri dish of pea-straw agar a little of the ground-up, fumigated pea straw is transferred with sterile forceps from the jar to a sterile Petri dish. Over the straw is poured 10 or 15 cc. of melted 1½ or 2 per cent water agar and the dish is then agitated in order to distribute the material evenly throughout the agar. The warm agar instantly drives off any residual fumigant which may still be present in the straw, whereupon organisms may then be cultured upon the medium as soon as it hardens. Where not combined with melted agar, additional time or warming may be required to dissipate residual gas from the straw.

Materials so fumigated, if not too moist, may be kept indefinitely in the closed containers without loss due to spoilage. Some plant materials prepared 1 and 2 years ago are still being used in this laboratory without resterilization.

Apparently no residue toxic to the growth of fungi is left in the tissue after sterilization and after dissipation of the fumigant. Furthermore, medium prepared from fumigated straw, for example, has proved superior to medium which has been made by autoclaving the pea straw with the water agar, probably because the nutrient contained in the straw largely remains in the pieces of tissue, apparently in a natural condition, and does not escape into the agar itself to any appreciable extent. This is a distinct advantage in that contaminants establish themselves with greater difficulty on a non-nutrient agar, whereas the fungi established in invaded substrates plated on this medium grow into the agar and reach the nutrient-containing pieces of pea straw.

What has been said here of pea straw applies likewise to other kinds of organic matter. Some 50 different kinds of plant and animal tissues, including scale and other insects, have been used in this way. Where fruiting of fungi is desired, the fumigated organic material may be sprinkled on the top of the water agar or more of it added at the time of pouring the plate.

Ethylene oxide because of its lower boiling point (10.7° C.) may best be handled in a low-temperature room. It is dissipated more rapidly from the material than is propylene oxide (boiling point, 33.9° C.) and is perhaps somewhat more efficient as a fumigant. However, since it is explosive in mixtures with air and more hazardous and difficult to use in the laboratory, the writers have preferred to use propylene oxide. Although inflammable, propylene oxide is easily handled. A small amount of it is kept in a stoppered bottle in a refrigerator held at about 7° C., ready for use at any time.

Gaseous sterilization has made it possible to prepare quickly and conveniently a variety of natural media without alteration by heat. The materials are collected and fumigated when available and stored in the jars until needed. The sterilization of limited volumes of soil, and of cultures of pathogenic organisms prior to their discard also has been carried out advantageously by this method. That soil may be sterilized with ethylene oxide has been reported by Roberts *et al.*[3]—H. N. HANSEN and WILLIAM C. SNYDER, Division of Plant Pathology, University of California, Berkeley.

*D-D Mixture as a Soil Treatment for Bacterial Wilt on Tobacco.*[1]—Commercial D-D mixture (dichloropropane and dichloropropene) was tested for control of bacterial wilt (*Bacterium solanacearum* E. F. Smith) of tobacco. The plots were 12 × 24 feet with water-tight sidewalls as described previously.[2] The material was placed in holes opened with a spade in the prepared soil March 8, 1944. No paper or water seal was used, although rain occurred on March 9 in an amount sufficient to wet the upper 2 inches of soil. A wilt-susceptible strain of tobacco was transplanted June 1, 1944.

Treatments at the rates of 0, 2, 4, and 8 ml. per square foot of soil surface had 82, 59, 24, and 0 per cent wilt, respectively, at the end of the season in 1944. The same plots replanted in susceptible tobacco without further treatment had 83, 71, 73, and 11 per cent wilt August 1, 1945. No toxic effects were observed although a dark green leaf color, brittleness, and upward roll of leaf margins typical of excessive chlorine occurred in 1944 in plots treated with the heavier rates.

D-D mixture gave considerable protection that persisted into the second successive tobacco crop as shown by the reduced amount of wilt in 1945 on the plot treated with 8 ml. per square foot. Some residual protection has been observed on soil treated with chloropicrin or urea, and on plots rotated to corn or other crops. However, the degree of protection from D-D mixture the second season was greater than with other treatments.—T. E. SMITH.

[3] Roberts, James L., L. E. Allison, Paul S. Prickett, and K. B. Riddle. Preliminary studies on soil sterilization with ethylene oxide. Jour. Bact. **45**: 40. 1943.

[1] Cooperative investigations of the Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, the N. C. Agricultural Experiment Station and Department of Agriculture.

[2] Smith, T. E. Control of bacterial wilt (*Bacterium solanacearum*) of tobacco as influenced by crop rotation and chemical treatment of the soil. U. S. Dept. Agr. Circ. 692. 1944.